1961

The Denaturation of Pepsin

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THE DENATURATION OF PEPSIN

by

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A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

June
1961

VITA

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The author is a member of the American Chemical Society, The Society of the Sigma Xi, and the Albertus Magnus Guild. He is a Co-author of the following publications:

Pairent, F. W. and Williamson, M. B., "The Tryptic Hydrolysis of Pepsin", Federation Proc., 18, 298 (1958).

White, J. W., Jr. and Pairent, F. W., "Report on the Analysis of Honey. Diastase Value of Honey", J.A.O.A.C., 42, 344-346 (1959).

Pairent, F. W. and Williamson, M. B., "A Modified Two-Dimensional Paper Chromatographic System for the Separation of DNP-Amino Acids", J. Chromatography, 4, 80-84 (1960).

Pairent, F. W. and Williamson, M.B., "Effect of Denaturation on the Chromatography of Pepsin", Federation Proc., 20, 239 (1961).

ACKNOWLEDGMENT

The kind encouragement of many relatives and friends has enabled me to accomplish this goal. I especially wish to express my sincere appreciation to the following:

To my parents, who lacked college degrees, but not the foresight and desire to guarantee mine.

To my wife, Constance, whose faith and assistance has been my most valuable asset.

To Dr. Martin B. Williamson, who has had the greatest influence on my academic accomplishments.

And to the faculty members of the Department of Biochemistry, whose confidence and guidance has often been greatly needed.

F.W.P.

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CHAPTER I

THE NATURE OF PROTEIN STRUCTURE

Proteins are complex organic nitrogenous compounds which are closely associated with all aspects of dynamic function constituting the life of an organism. They serve as enzymes, hormones and structural components of cells. They are associated with the hereditary factors and with the immunological defense mechanism. There is hardly a single physiological function in which proteins do not participate. The ultimate knowledge of biochemical processes will undoubtedly depend on an understanding of the activity of proteins.

The structure of a protein is the determinant of its activity. A specific and unique arrangement within the construction of a protein confers upon the molecule the ability to act in a relatively specific and unique manner. The ultimate goal of the protein chemist is to correlate this structural arrangement with the activity of the protein. Numerous properties of proteins can be used to investigate its structure. One of the most interesting and complex classes of reactions of proteins are the changes which can take place when protein

molecules are removed from their native environment. These changes are frequently referred to as "denaturation". A knowledge of these changes is important because of the information it can provide about the more intimate details of protein structure and function.

I. DENATURATION OF PROTEINS

The most basic definition of denaturation is given as "the loss of original properties". When applied to proteins, this definition is found to be quite inadequate. At the present time there is no general agreement on a precise meaning of the word "denaturation" with regard to macromolecules. It has been used to describe a variety of reactions of proteins, and has thereby generated a feeling of vagueness about a phenomenon which indubitably does exist.

Kauzmann (42) has interpreted denaturation as "a process (or sequence of processes) in which the spatial arrangement of the polypeptide chains within a molecule is changed from that typical of the native protein to a more disordered arrangement". This definition was offered only to describe the phenomenon that occurs when a protein is subjected to "denaturation". It indicates three things:

(1) that native proteins have a definite configuration;

(2) denaturation causes this configuration to change;

(3) this change due to denaturation results in a more

disordered arrangement of the polypeptide chain.

The changes which occur during denaturation have been divided into two general classes: (1) changes in "shape" properties and (2) changes in "short range" properties (42).

A "shape" property is an indication of the molecular weight and the overall dimensions of the molecule. An alteration in either of these molecular parameters may be detected by a change in the sedimentation and diffusion constants, viscosity, and light scattering properties of the protein, among others.

Changes in "short range" properties indicate an alteration in the immediate vicinity of individual groups in the molecule. Such properties include: (1) thermodynamic properties; e.g., heat capacity, entropy, free energy, solubility and activity; (2) optical properties; e.g., optical rotation and dispersion, light absorption, x-ray diffraction; (3) chemical properties; e.g., reactivity of groups, intrinsic pK's of acidic and basic groups, capacity to bind small molecules, dyes, ions, etc., digestability by proteolytic enzymes, biological activity, electrophoresis and chromatography.

Many, if not all, of the properties of a native protein indicated above can be demonstrated to undergo a change when "denaturation" occurs. Several reviews have been published concerning the detection and measurement of changes in

these proterties during denaturation (41, 42, 53, 75). To review the entire subject here would be outside the scope of this dissertation. However, several of the properties listed will be discussed in the next chapter, with respect to the results obtained for pepsin.

II. MAINTENANCE OF PROTEIN STRUCTURE

Linderstrom-Lang (47) has proposed a classification of protein structure that includes all types of bonding forces which are thought to exist in native proteins. He speaks of the "primary", "secondary", and "tertiary" structure of a protein molecule. The sequence of amino acids along the polypeptide chain, and the location of disulfide bonds in relation to this sequence, is referred to as the "primary" structure. The "secondary" structure is the spatial configuration of the polypeptide chain which is maintained by the hydrogen bonding between the peptide N - H and C = O groups. The α -helix and β -sheet structures of Pauling, et al (62), are illustrations of possible "secondary" structures for polypeptides. The "tertiary" structure is the pattern according to which the "secondary" structures are arranged within the native protein molecule.

Not much evidence is, as yet, available on the "secondary" and "tertiary" structure of proteins. Therefore,

the distinction between "secondary" and "tertiary" structure of a protein must be regarded as tentative. It is by no means certain that the structural patterns resulting from hydrogen bonding between peptide links ("secondary" structure) are inherently stable and can exist apart from the stabilization brought about by other types of intramolecular bonds ("tertiary" structure).

A. Primary Structure

It has long been established that a protein molecule is constructed of a chain(s) of α-amino acids bound together by peptide bonds between amino and carboxyl groups. Three types of polypeptide chains are theoretically possible: straight chain, cyclic chain and branched chain. These are illustrated by the following structural formulae:

Straight Chain

$H_2N.RCH.CO-NH.R''CH.CO.....NH.RCH.CO-NH.R'CH.COOH$

At one end of the polypeptide is found an amino acid having a free α-amino group (N-terminal); at the other end of the peptide is found an amino acid having a free carboxylic group α- to the amino group (C-terminal).

Cyclic Chain

....NH.RCH.CO.NH.R'CH.CO....
.   .
....

These polypeptides have neither N nor C-terminal groups.

Branched Chain

H_2N.RCH.CO....NH.CH.CO...NH.R'CH.COOH
$(CH_2)_4$
HN.OC.CHR'.NH....OC.CHR.NH_2

These polypeptides contain more of the one type of terminal amino acid than of the other. The illustration shown has two N-terminals and one C-terminal.

Combinations or multiples of these three basic forms are, of course, possible. The first two forms illustrated have been found to be the most common.

Some proteins are built up of two or more polypeptide chains, held together by a stable bond other than a peptide link. Such a bond is referred to as a "cross linkage". The disulfide bond of cystine is the most common type of "cross linkage". Here, two cysteine residues are joined together (S-S) through their side chains. The less common phosphodiester bond is a

much weaker link, and has been found in only a few proteins (65).

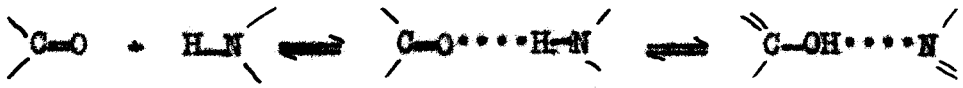
The amino acids of the polypeptide chain are arranged in a unique sequence which theoretically endows the molecule with potential activity. This activity is only realized when the polypeptide is patterned into a certain specific configuration. The configuration of proteins is maintained by "secondary" and "tertiary" bonding.

B. "Secondary" and "Tertiary" Structure

Before an interpretation of experimental observations on protein denaturation can be made, the nature and characteristics of the intramolecular bonds which stabilize the native form of the protein must be considered. These are the bonds which contribute to the "secondary" and "tertiary" protein structure. It is the rupture of these bonds, leading to the disordering of the native protein structure, which is the characteristic feature of denaturation.

(1) Hydrogen bonds between peptide links: The hydrogen bonds between the oxygen atoms of the carboxyl groups and the hydrogen atoms of the amide groups of peptide linkages are assumed to play a basic role in determining the pattern of coiling of polypeptide chains in most of the structures which have been proposed to account for the x-ray diffraction pattern of fibrous proteins (5, 37, 62, 76). There is every reason to believe that this bond is also an important factor in many

globular proteins, if not all of them. An extensive treatment of the hydrogen bond is given by Pimentel and McClellan (72). The peptide hydrogen bond can be illustrated as follows:



(2) Hydrogen bonds between side chains: Various types of hydrogen bonds other than those between peptide links are certainly possible in proteins. Considerable attention has been directed to hydrogen bonds involving the phenolic hydroxyl of tyrosine as contributing to the stability of the "tertiary" structure of proteins. Tyrosyl-carboxylate bonds are believed to exist in insulin (45), ribonuclease (82), ovalbumin (15), and pepsin (9), as well as others. Wetlaufer (92), using a simple model system, deduced that the tyrosine-carboxylate hydrogen bond can be no stronger than the hydrogen bond between two peptide groups, and that it might well be weaker.

Laskowski and Scheraga (44) have presented an orderly classification of side chain hydrogen bonds based on the type and number of interacting groups, including ionized groups as one possible partner in the hydrogen bond. They have suggested an unusual hydrogen bond between two carboxyl groups. Carboxyl-carboxyl hydrogen bonds would, of course, only be expected at acid pH. Edelhoch (20, 21) has suggested the presence of such

hydrogen bonds in pepsin.

(3) Hydrophobic bonds: Nearly all proteins contain a relatively high proportion of amino acids with non-polar side chains, such as valine, the leucines, and phenylalanine. In the great majority of proteins, the amount of non-polar amino acids approximate 50 per cent. Since the non-polar side chains have a low affinity for water, those polypeptide chain configurations which bring large numbers of these groups into contact with each other, will be more stable than other configurations. The non-polar groups will have a tendency to adhere to one another in aqueous environments. This tendency is referred to as "hydrophobic bonding". The hydrophobic bond is probably one of the most important factors involved in stabilizing the "tertiary" configuration of many native proteins (26, 42).

(4) Salt linkages: (Electrostatic forces) It has been suggested that an important factor in maintaining the structure of native porteins is the electrostatic attraction between the positively charged amino and guanidino groups and the negatively charged carboxyl groups that are so abundant in proteins. Although it is quite possible that some of these bonds may be present in certain proteins, Jacobsen and

Linderstrom-Lang (38) have given various reasons for believing that only a small fraction of the charged groups can be involved in bonding of this kind.

Both salt linkages and hydrophobic bonds are stabilized predominantly by entropy effects rather than by energy effects (81). The two types of bonds would be expected to respond very differently both to the addition of electrolytes to the aqueous medium, and to the addition of non-polar substances. Non-polar substances should weaken hydrophobic bonding, while they strengthen salt linkages. Electrolytes should tend to strengthen hydrophobic bonds, and weaken salt linkages. It is commonly observed that solvents such as dioxane, alcohols, and acetone, act as strong denaturing agents toward proteins, whereas electrolytes are inhibitors of denaturation. These observations would seem to show that hydrophobic bonds are much more important than salt linkages in maintaining the native configuration.

Other factors that may possibly contribute to the stability of "tertiary" structure include electron delocalization (4, 6, 31) and dispersion forces, or van der Waal's forces.

The following tentative conclusions can be drawn concerning the forces responsible for maintaining the native

configurations of proteins:

(1). The denaturing action of urea analogues on proteins is usually considered to be due to their ability to attack hydrogen bonds.

(2). The irreversible inactivation of enzymes brought about by increasing or decreasing the hydrogen ion concentration may be indicative of the involvement of hydrogen bonding between side chain groups of the protein, where one group of the hydrogen bond pair is an ionizable group.

(3). The ability of detergents and organic solvents to denature proteins indicates the importance of hydrophobic bonds, since they would be weakened by these reagents.

(4). The ineffectiveness of electrolytes as denaturants is an indication that salt linkages may not be general contributors to the stability of proteins.

(5). Because of the large number of peptide groups and hydrophobic groups found in proteins, it is likely that hydrogen bonds between two peptide links, and hydrophobic bonds are by far the most important determinants of the overall configuration of the protein molecule. Yet the activity of proteins, especially enzymes, in all probability depend on the configurations present in localized regions of the molecule ("active centers"). These "active center" configurations might very well be determined by some of the less abundant types of

bonds. Because of this, none of the bonds discussed above can be considered less important than the others.

CHAPTER II

THE DENATURATION OF PEPSIN

The results of a study on the denaturation of pepsin will be presented in this dissertation. In order to fully evaluate the results found, a review of pertinent data from the literature is essential. The known chemical and physical characteristics of pepsin will be presented in the first section of this chapter, so that a background for the review will be established.

I. CHEMICAL AND PHYSICAL CHARACTERISTICS OF PEPSIN

Pepsin is the major proteolytic enzyme found in the stomach of all mammals. It is secreted in an inactive precursor form, pepsinogen, by the glands of the gastric mucosa (31). The activity of pepsin is characterized by the rapid hydrolysis of peptide bonds involving the amino group of tyrosine and phenylalanine (6, 8).

The enzyme was first crystallized from commercial preparations of swine pepsin by Northrup (56). Purification is accomplished by precipitation with magnesium sulfate at pH 2,

dissolution of the precipitate by addition of alkali to a pH slightly less than 4, and reprecipitation by acidification. Crystallization occurs when this precipitate is dissolved at pH 4, 45°, and the solution allowed to cool. Pepsin may also be crystallized from 20 per cent ethanol and is soluble in 65 per cent ethanol (55).

Pepsin is produced autocatalytically from its precursor upon acidification of pepsinogen solutions (31, 32). Six peptide bonds are hydrolyzed in the precursor to produce pepsin plus six small peptides. The largest of the peptides has been found to inhibit the activity of pepsin above pH 5.4, by forming a pepsin-inhibitor complex. At a pH below 3.5, the inhibitor is hydrolyzed by pepsin.

The chemical characteristics of pepsinogen, pepsin and pepsin inhibitor have been determined and are shown in Table I. From the experimental data outlined in this table, it has been assumed that pepsin and pepsinogen are both single polypeptide chains (2).

The amino acid compositions of pepsinogen (90), pepsin (10, 12), and pepsin inhibitor (91) are shown in Table II.

A unique structural feature of the pepsin molecule, and also of pepsinogen, is the presence of a single phosphate diester bridge (63, 65). The phosphate can be removed enzymat-

TABLE I

THE CHEMICAL CHARACTERISTICS OF PEPSINOGEN AND ITS DERIVATIVES

	PEPSINOGEN	PEPSIN	INHIBITOR
N-terminal sequence	*Leu.Leu- (33,90,10,57)	*Ileu.Gly.Asp.Asp- (10,28,33,60,93)	*Leu.Glu- (91)
C-terminal sequence	-Val.Leu.Ala* (90)	-Val.Leu.Ala* (33,61,94)	unknown
Phosphorus atoms / mole	1 (24)	1 (31)	0 (91)
Half-cystines / mole	6 (43)	6 (43)	
Lysines / mole	12 (90)	1 or 2 (10) (12)	4 (91)
Molecular weight (a)	42,862 (90)	35,298 (10,12)	3,242 (91)
Molecular weight (b)	42,500 (57)	34,500 (16,43,52,54,57,70)	3,100 (32)
Isoelectric point	3.7 (56)	less than 1 (56)	

* terminal amino acid residue
(a) sum of analytical values for amino acid analyses
(b) sum of physical measurement values

TABLE II

AMINO ACID COMPOSITION OF PEPSINOGEN, PEPSIN AND INHIBITOR

Amino Acid	Residues per mole			
	Pepsinogen (90)	Pepsin (12)	(10)	Inhibitor (91)
Aspartic acid	46	41	44	4
Glutamic acid	32	28	27	2
Glycine	36	29	38	1
Alanine	27		18	2
Valine	27	21	21	2
Leucine	64	27	28	5
Isoleucine		28	27	
Serine	53	40	44	2
Threonine	25	28	28	1
Half-cystine	6	4	6	
Methionine	5	4	5	
Proline	20	15	15	3
Phenylalanine	20	13	14	1
Tyrosine	16	16	18	1
Tryptophane	4	4	6	
Histidine	4	2	1	
Lysine	12	2	1	4
Arginine	3	2	2	1
amide NH_3	39	32	36	
Total amino acids	400	304	343	29

ically by potato phosphatase at pH 5.6; the dephosphorylated pepsin is fully active. Perlmann (65, 66) suggested that the structural function of this diester bond is to link two distant sites of the single peptide chain into a loop. As yet, only one point of attachment of the diester bond has been determined. Flavin (24, 25) has provided evidence that the phosphate group in pepsin is esterified to a serine residue in the sequence: -threonyl-serylphosphate-glutamyl-.

Several investigators have employed various chemical reactions with pepsin in an effort to determine the type of reactive groups that contribute to peptic activity. The work of Herriott has implicated the involvement of carboxylic groups and tyrosine residues as necessary for peptic activity; free amino groups were not found to be essential (29, 30, 34). Kern (43) reported that the reduction of one disulfide bond out of the three found in pepsin, does not impair enzymic activity. Reduction of more than one disulfide bond results in the rapid loss of enzymic activity.

II. THE HETEROGENEITY OF PEPSIN PREPARATIONS

Pepsin appears to be the least stable of all proteolytic enzymes. Purification of the native enzyme must be performed in acid solutions because exposure of pepsin to solutions more alkaline than pH 6 results in rapid loss of

peptic activity (56). Since pepsin has the ability to digest itself in acid solutions, the separation of the active enzyme from its autodigestion products is extremely difficult. Thus, many investigators have reported anywhere from five per cent to 20 per cent "split products" present in various pepsin preparations. In all cases, the amount of "split products" was estimated from the absorption at 280 mu of the trichloroacetic acid soluble peptide material.

Electrophoresis of crystalline pepsin preparations by Tiselius, et al (89), gave one sharp, and a second diffuse boundary which was visible at high concentration only. Hoch (36) subjected a sample of crystalline pepsin to electrophoresis at pH 3.9, 5.9, and 8.0. His results were similar to those of Tiselius, and the pepsin appeared to be about 96 per cent pure. However, prolonged electrophoresis (28 hours) at all three pH's indicated that the major peak was heterogeneous. The pattern at pH 5.9 clearly showed the presence of four components.

Several investigators have shown the chromatographic heterogeneity of commercial pepsin preparations, and have reported the presence of other proteolytic enzymes associated with pepsin, either in gastric juice or in extracts of the gastric mucosa. Freudenberg (27), Pope and Stevens (73), Buchs (13), and Taylor (88) have shown that components may be found with optimum activity at pH values above 3.0. This activity

has generally been attributed to a cathepsin (87). Merten, et al (49), by precipitation and electrophoresis, and Richmond, et al (77), by passage through a cation exchange resin, obtained partial separations of the fractions with optimum activity below pH 2.0 and above 3.0.

Ryle and Porter (78) fractionated crude pepsin by ion exchange chromatography on diethylaminoethyl-cellulose and have isolated two minor components which differ from pepsin in enzymatic specificity but are similar to it in other properties. These authors named the enzymes Parapepsin I and II. A comparison of the properties of the Parapepsins and pepsin are presented in Table III. Ryle and Porter tested three batches of commercial crystalline pepsin by chromatography. No Parapepsin II was found in any of them, but one batch contained Parapepsin I, accounting for four per cent of the total activity against the synthetic substrate acetylphenylalanyl-di-iodotyrosine. Ryle (79) later reported isolation of the zymogen of Parapepsin II: Parapepsinogen II. The similarity of the zymogen to pepsinogen reflected the similarity of the enzymes.

The determination of the N-terminal amino acids of a protein preparation is considered to be a sensitive criterion of protein purity. Analysis of the N-terminal amino acid is best accomplished by the dinitrofluorobenzene

TABLE III

PROPERTIES OF PEPSIN AND PARAPEPSINS I AND II (78)

	Parapepsin I	Parapepsin II	Pepsin
Substrate for assay	APD*	Hb*	Both
m [P.U.] mg	40	35	23
Sedimentation ($S_{20,w}$)	3.26	3.32	3.16 (19)
Molecular Weight	38,600	40,700	34,500 (90)
N-terminal amino acid	Ala	Ser+Leu/Ileu**	Ileu (28,90)
Phosphorus (g. atom/mole)	0.03	0.0	1.0 (54)
Stability at pH 6.9	Stable	Unstable	Very unstable (83)
pH optima	1.8	1.8, 3.0	1.5-2.0
Digestion of bovine albumin	+	+	++++
[P.U.] (milk clotting)/ [P.U.]	0.12	0.47	1.2
Inhibition by pepsin inhibitor	-	+	+ (32)
[P.U.] (gelatin) / m [P.U.]	12.1	2.5	0.15

*APD = acetyl-dl-phenylalanyl-l-di-iodotyrosine
Hb = hemoglobin

**Isomer not identified.

(DNFB) method (74, 80), or by reaction of the protein with phenylisothiocyanate (PTC) (22, 23). The N-terminal amino acid of pepsin has definitely been determined by both methods to be isoleucine (10, 28, 33, 93). Other N-terminal amino acids, detectable in small amounts, have been reported to be present in crystalline pepsin preparations. These N-terminal amino acids have generally been attributed to the presence of the products of autodigestion. Heirwegh and Edman attempted to purify a crystalline pepsin preparation by cation exchange chromatography (28). They found that they could increase the specific activity of the pepsin by more than 25 per cent, but the contaminating N-terminal amino acids were still present.

III. THE STABILITY OF PEPSIN

Results found in the literature which are most pertinent to the investigation reported in this dissertation will be presented in this section. It should be emphasized that when dealing with the actual changes that occur during protein denaturation, the term "denaturation" becomes operational. That is, "denaturation" is now defined in terms of the experimental criteria which is applied to measure the actual change taking place. It might be well to have two separate terms, since it seems advantageous to speak separately of the "process" of denaturation, and the actual "denatured state" of a protein.

However, the introduction of another term at this point, might well add to the confusion, instead of avoiding it.

The definition of denaturation given in Chapter I was meant to be applied to the "process" of denaturation. When we measure the "degree" of denaturation as caused by the "process", we are implying that a certain amount of the protein has been changed to a "denatured state". Thus, we may speak of denaturation in terms of a particular experimental property, such as the solubility in water at the isoelectric point, or the loss of some typical biochemical characteristic possessed by the protein (enzymatic activity, ability to combine with antibodies, etc.).

The operational type of approach might well be preferred, but it has the disadvantage that definitions in terms of solubility or activity draw attention away from the most significant aspect of the phenomenon, namely its intimate relationship to protein structure. Hence, changes in solubility or activity might not be indicative of "a change of spatial configuration to a more disordered arrangement".

A. Alkaline Inactivation of Pepsin:

It is known that the enzymatic activity of pepsin is rapidly lost in weak alkaline solutions. This inactivation has been termed the "alkaline denaturation" of pepsin. Northrup

showed that the alkaline inactivation of pepsin was accompanied by the formation of acid-insoluble protein, which he termed "denatured pepsin" (56). His results are summarized in Table IV. The pepsin solution was titrated to a series of pH between 5.6 and 8, and then acidified. The resulting precipitate was filtered, and the supernatent was tested for enzymatic activity and analysed for its nitrogen content.

TABLE IV

EFFECT OF ALKALI ON THE ENZYMATIC ACTIVITY OF PEPSIN AT 20°. (56)

pH	% Activity	% Soluble N
5.6	100	100
6.5	90	89
7.0	70	67
7.5	26	23
8.1	1	--

Michaelis found that the rate of inactivation of pepsin was proportional to about the fourth power of hydroxyl ion concentration between pH 6.0 - 8.0 (50). Steinhardt reported that the rate of inactivation is inversely proportional to the fifth power of the hydrogen ion concentration in this

range (83).

Edelhoch determined the titration curves of both native and denatured pepsin (18, 20). He found that when pepsin is subjected to alkaline inactivation, protons are released into the solution and new acid-binding groups can be titrated in the denatured protein. The liberation of protons and the inactivation of pepsin were found to proceed at approximately identical rates. The acid liberated amounted to 5 - 6 moles of hydrogen ion per mole of protein (18). The alkaline pH limit of pepsin stability was found to vary with the temperature (20) as follows:

Temperature	pH
31°	6.00
25°	6.20
12°	6.85

The stability of the enzyme was also found to decrease with an increase in ionic strength of the solution, or with an increase in ethyl alcohol concentration.

The presence of small amounts of metal ions were found to increase the rate of denaturation of pepsin between pH 6.3 - 6.5 (17). These metal ions, in decreasing order of effectiveness were: Cu^{++}, Pb^{++}, Cd^{++} and Zn^{++}. Other metal ions

(Hg^{++}, Ag^{++}, Mn^{++}, Co^{++}, Mg^{++}, Ni^{++}, and Fe^{++}) had little or no effect at comparable concentrations. The relative effectiveness of these metals parallel the order of their affinity constants for carboxyl groups, with the exception of Hg^{++}. Edelhoch concluded that the type of weak bond that appears to conform best with all the data is a hydrogen bond found between a carboxyl group acting as a donor and possibly a second carboxyl group acting as an acceptor.

In another report, Edelhoch evaluated the effects of urea, metallic cations, ethyl alcohol, trimethyl dodecylammonium chloride and several combinations of these reagents on the enzymatic stability of pepsin by means of kinetic inactivation experiments (21). He reaffirmed his previous assumption that pH, urea and specific metallic cations modified the stability of pepsin by their effect on carboxyl (donor) hydrogen bonds. He further concluded that ethyl alcohol and trimethyl-dodecylammonium chloride disturbed the hydrophobic intramolecular bonding of the "tertiary" structure which brought about a decreased stability of the carboxyl hydrogen bonds.

Pepsin apparently undergoes gross changes in the mass and shape of its molecular kinetic unit during denaturation by alkali (18, 19). This is demonstrated in Table V, which lists the molecular parameters of both native and denatured pepsin.

TABLE V

"SHAPE" PROPERTIES OF NATIVE AND DENATURED PEPSIN (19)

	Native (pH 6.0)	Denatured (pH 7.0)
Sedimentation ($S^{\circ}_{20,w}$) x 10^{13}	3.20	2.17
Diffusion ($D_{20,w}$) x 10^{7}	8.71	6.9
Molecular Weight x 10^{-3}	35.8	30.7
Frictional Ratio (f/f_o)	1.11	1.48
Axial Ratio (a/b)	3.0	8.8

The effect of pH on the sedimentation coefficient of pepsin was measured from pH 1.2 to 10.2. From pH 1.2 to 6.0, the sedimentation coefficient was independent of pH. The schlieren patterns in acid media showed a single, symmetrical boundary. Solutions of alkali denatured pepsin also showed only a single boundary in the ultracentrifuge, and no effect of pH on the sedimentation coefficient was observed from pH 7.0 to 10.2. Acidification to pH 6.0 of a solution of denatured pepsin had no influence on the sedimentation characteristics that were observed at pH 7.0. The changes in the sedimentation coefficient produced on denaturation are, therefore, not reversed with pH.

Combined with the diffusion data, the sedimentation coefficient of denatured pepsin indicates a decrease in molecular

weight of pepsin. Viscosity and light scattering measurements are compatible with this suggestion (19). Furthermore, the electrophoretic diagram obtained with denatured pepsin solutions (from pH 6.5 to 9.6) indicate at least three, and probably more, components are present. Analysis of the various schlieren peaks indicated that the largest component constituted about 75 per cent of the total composition.

B. Effect of Denaturants

Steinhardt has shown that if pepsin is stored in 1.0 to 6.0 M urea (acidic solutions) at 3° for one to fifteen days, the proteolytic activity is retained, and the sedimentation coefficient of the protein remains constant (84). Perlmann found that pepsin activity is lost under the same conditions when the temperature is raised above 20° (66). Table VI shows the marked loss of enzymic activity when pepsin is exposed to 8.0 M urea, pH 4.6 at 25° and 37°.

Blumenfeld, et al, reported that guanidine hydrochloride acted on pepsin in a manner similar to urea (11). It was shown that on prolonged exposure to guanidine hydrochloride, pepsin is irreversibly inactivated. In the pH range of 1.0 to 4.4 the loss of activity is accompanied by the appearance of low molecular weight peptides. It was concluded that these peptides are a result of the autodigestion of the protein. The results

TABLE VI

EFFECT OF TEMPERATURE ON
THE INACTIVATION OF PEPSIN IN 8 M UREA, pH 4.6

Time (hr.)	Relative Activity		
	3°	25°	37°
0	100%	100%	100%
2	--	89	50
4	--	80	24
6	--	74	11
24	100	40	0

of these experiments are shown in Table VII. The formation of trichloroacetic acid (TCA) soluble material was also found to parallel pepsin inactivation in 8.0 M urea solutions. These observations were summarized as follows (11):

1) The rate of inactivation of pepsin in 8.0 M urea solutions was slowest in the range from pH 3.3 to 4.3 (less than 5 per cent inactivation in one hour at 37°). In 4.0 M guanidine hydrochloride solutions, this range of maximum stability was from pH 3.0 to 3.5 (less than 10 per cent inactivation in one hour at 37°).

2) The loss of pepsin activity in the presence of urea

TABLE VII

EFFECT OF GUANIDINE HYDROCHLORIDE ON ENZYMATIC ACTIVITY OF PEPSIN

	3.0 M Guanidine HCL		4.0 M Guanidine HCL	
Apparent pH	Inactivation	TCA Soluble Material	Inactivation	TCA Soluble Material
1.2	55%	45%	100%*	--
2.0	10	19	83	62
2.4	--	--	14	15
3.1	1	3	9	14
3.5	1	3	9	6
4.1	--	--	25	27
4.4	9	7	63	60
5.0	100 *	49	--	--

* Complete inactivation after 30 minutes.

Reaction mixtures at 37° for one hour.

or guanidine hydrochloride proceeds rapidly below pH 3.0, and the formation of low molecular weight peptides closely parallels the rate of inactivation. This was considered to be the result of the action of intact pepsin on denatured molecules.

3) Pepsin is known to lose its activity spontaneously, without the formation of non-protein nitrogen at pH values greater than 6.0. If guanidinium ion or urea is present in the reaction mixture, this rapid loss of activity will occur at a more acid pH value.

Perlmann performed electrophoretic analysis on a solution of pepsin that was incubated at 37° in 8 M urea-acetate buffer at pH 4.6, and then dialysed (66). The electrophoretic patterns demonstrated the formation of two new (non-dialysable) components, at the expense of the original (active) protein. The electrophoretic components were labeled "P", "P_1" and "P_2", in decreasing order of mobility at pH 4.6. The composition of the electrophoretic patterns are given in Table VIII. The "P" component was the only component with activity (toward hemoglobin). Even after complete inactivation, material is still present with an electrophoretic mobility and molecular weight (ultracentrifuge measurement) similar to that of the enzymatically active material. In later work (67), pepsin was treated with 8.0 M urea, at 37° for 24 hours, dialysed, and subjected to

TABLE VIII

ELECTROPHORETIC ANALYSIS OF PEPSIN SOLUTIONS AT DIFFERENT STAGES OF UREA INACTIVATION*

Time (hr.)	Relative Specific Activity per unit Weight	Electrophoretic Composition		
		"p"	"P_1"	"P_2"
0	100	100%	--	--
6	51	88	7%	5%
12	26	75	14	11
18	15	67	17	16
24	8	63	20	17
42	0	37	39	24

*pH 4.6, 8.0 M Urea, 37°

zone electrophoresis on Geon resin. The active component isolated from the resin appeared to be somewhat different from the original pepsin. The sedimentation constant was only slightly lower (S_{20} = 2.96 x 10^{-13} for pepsin; S_{20} = 2.81 x 10^{-13} for the active component), but the relative specific activity per unit nitrogen of the modified protein was 40 to 50 per cent higher than that of the original pepsin. If the activity was expressed per unit tyrosine, the increase in specific activity was 80 to 100 per cent. Preliminary amino acid analysis revealed

differences in the tyrosine content, as well as in several other amino acids.

There is one other report dealing with the formation of a modified pepsin enzyme (64). Perlmann found that proteolytic activity can be recovered in the non-protein fraction resulting from the 48 hour autodigestion of pepsin at pH values above 2.5. When crystalline pepsin is incubated at 37°, pH 5.6, for 24 hours, and then dialysed 24 hours at a low temperature, the dialysate is found to contain one to five per cent of the original pepsin activity when hemoglobin is used as the substrate. When a synthetic dipeptide substrate (acetyl-phenylalanyl-di-iodotyrosine) is used, the relative specific activity per unit nitrogen of the dialysate is found to be 64 per cent of the original solution. This work was published in 1954. No further extension of this work has been reported since.

Only one other report in the literature concerning pepsin is of importance in this summary. This paper concerns the effects of solvents on the optical rotatory properties of pepsin (68). Most globular proteins have a specific rotation, $[\alpha]_D$, in the range from -30° to -70°. which on denaturation with urea or guanidine salts becomes more negative by 20° to 60° (35, 40). Similarly, the optical rotatory dispersion constant, λ_c, is lowered from the range of 230 - 270 mu upon

contact with these reagents (90). A protein having a λ_c of 212 mμ is considered to be in randomly coiled form devoid of any helical structure (14, 96). A higher value of λ_c indicates that a certain fraction of the amino acid residues of the polypeptide chain are involved in helical configuration.

The specific rotation of pepsin, $[\alpha]_{600} = -63.5 \pm 0.5°$, (pH 3.5 - 5.6), was found to be within the range of other globular proteins, but the dispersion constant, λ_c, was found to have a value of only 216 mμ (39, 68). When the protein is dissolved in 8.0 M urea, pH 5.4 at 37°, the specific rotation remains constant, but λ_c decreases from 218 mμ to 202 mμ in 24 hours, while the activity drops from 95 per cent to 10 per cent of the original. If the solution is then dialysed, the constant of the non-dialysable fraction is found to be 210 mμ, and the specific activity is increased to 66 per cent of the original. The value of 202 mμ for λ_c was attributed to the presence of peptide material. (The value of 212 mμ was calculated for proteins.) It was concluded that pepsin solutions at 20° - 25° do not contain appreciable amounts of protein with helical configuration.

CHAPTER III

EXPERIMENTAL PROCEDURE

In a previous investigation, the hydrolysis of pepsin by trypsin was studied in an attempt to obtain peptides of pepsin which might be amenable to amino acid sequence studies. Trypsin is believed to be specific in hydrolysing only those peptide bonds involving the carboxylic groups of arginine and lysine residues of proteins (8). According to amino acid analysis, pepsin contains two arginine and either one or two lysine residues (10, 12). Therefore, the tryptic hydrolysis of pepsin would be expected to result in the formation of three or four new N-terminal amino acids. Contrary to this expectation, it was found that the enzymatic hydrolysis of pepsin by trypsin gave rise to approximately 13 new N-terminal amino acids (58, 58a), as determined by the DNFB method (80). The results are summarized in Table IX. The N-terminal amino acid analysis prior to tryptic hydrolsis is also given.

The data shown in Table IX appears to indicate that trypsin does not have the usual specificity attributed to it. For this reason, it was essential to determine if the appearance

TABLE IX

N-TERMINAL AMINO ACID RESIDUES

FOUND AFTER THE TRYPTIC HYDROLYSIS OF PEPSIN[a]

	Residues / mole pepsin		
Amino Acid	"0" hours	72 hours[b]	Total[c]
Aspartic and Glutamic Acids	0.3	3.0	3
Leucine and Isoleucine[d]	0.9	2.4	3
Valine	0.2	0.9	1
Alanine	0.2	1.2	1
Glycine	0.2	2.7	3
Threonine	0.1	1.2	1
Serine	0.2	1.9	2
Total	2.1	13.3	14

a) Hydrolysis of 0.56 gm% pepsin preparation (corrected for ash and moisture) in 0.20 M phosphate buffer at pH 8.0 and at 37° C. (0.01% trypsin).
b) Corrected for zero hour results.
c) The nearest integer of residues of N-terminal amino acid per mole of pepsin.
d) Isoleucine is the N-terminal amino acid of pepsin.

of new N-terminal groups was due to some cause other than tryptic hydrolysis. No evidence was found to show that hydrolysis could have been caused by a proteolytic contaminant in the trypsin preparation. Therefore, the pepsin preparation itself became suspect. It was certain that the pepsin sample used was fully inactivated at the pH of the hydrolysis experiments. The possibility existed, however, that the pepsin sample contained contaminating peptides which might be hydrolysed in accordance with the known specificity of trypsin. It was believed that the N-terminal amino acids (other than isoleucine) detected by the DNFB method in an untreated pepsin sample were from the peptides arising from autolysis of the enzyme. However, these peptides might also have originated from some other source, and have an amino acid composition quite different from that of pepsin. To study this possibility, investigation of the heterogeneity of pepsin was undertaken. During the course of this investigation, certain anomalies were found which appeared to indicate that the heterogeneity of pepsin may be due to denaturation. It was then decided to undertake a more complex study of the effect of denaturation of pepsin on the formation of new peptides. This is the subject matter of the present dissertation.

I. DESIGN OF THE PRESENT EXPERIMENTS

The effect of urea and alkali on the inactivation of

pepsin was studied by chromatography on diethylaminoethyl-(DEAE)-cellulose columns. Pepsin was incubated at pH 5.5 in 8 M urea-acetate, 37°, for varying lengths of time and then chromatographed. The 280 mμ absorption of the effluent was taken as an indication of the protein concentration. The gross changes that occurred in the chromatographic patterns were compared with the changes occurring when the native enzyme is allowed to digest itself in acid solutions in the absence of urea.

The "alkaline denaturation" of pepsin (i.e., inactivation of the enzyme in solutions above pH 6) was followed in an identical manner. Pepsin was inactivated slowly at pH 6.7 and 7.0, 23°, before chromatography. The resulting chromatograms are compared with those obtained from urea inactivated samples. Various qualitative characteristics of the protein formed in both cases have been determined, and an explanation of the observations will be offered.

II. MATERIALS AND METHODS

The crystalline pepsin preparation used in these experiments was of porcine origin (Armour, lot no. 108-145). It was prepared by the method of Northrup (56), and recrystallized three times. The preparation was found to contain 6.5 per cent moisture, 2.7 per cent ash, and 14.4 per cent nitrogen. The homogeneity of the preparation was tested by three criteria:

1) electrophoresis, 2) constant solubility and 3) solubility in ten per cent trichloroacetic acid.

1) Electrophoretic analysis at pH 4.3 in acetate buffer (ionic strength = 0.1) indicated that approximately 96 per cent of the protein migrated essentially as a homogeneous protein.

2) A solubility curve of the pepsin preparation in 1.5 M $MgSO_4$, pH 5.0 acetate buffer indicated the presence of proteinaceous impurities amounting to approximately seven per cent of the total protein (58).

3) About 94 per cent of the total protein was found to be insoluble in ten per cent trichloroacetic acid (TCA), as measured by absorption at 280 mu.

The TCA solubility data only indicates the presence of low molecular weight impurities. The electrophoretic analysis only indicates the presence of high molecular weight impurities. On the basis of this data, approximately 80 per cent of the pepsin preparation appears to be homogeneous protein, if corrections are made for ash and moisture content.

All of the experiments reported were performed with the Armour pepsin sample. However, another preparation of porcine pepsin (Worthington, lot no. 632, 3 X crystalline) was employed to make certain that the results obtained were not unique to the Armour pepsin preparation.

A. Chromatography of Pepsin

The chromatographic system used in these experiments is a modification of the system reported by Ryle and Porter (78). Diethylaminoethyl-(DEAE)-cellulose was obtained from Brown Company, Berlin, N.H., and had about one milliequivalent of basic groups per gram. About 2 gm. of the ion-exchange material was suspended in distilled water and poured into a glass column, 0.9 cm. in diameter. It was then equilibrated with 0.10 M acetate buffer, pH 5.5, until the effluent solution had a pH of 5.5. The column was equilibrated under the same constant hydrostatic pressure that was to be used during the chromatography of the pepsin preparations. Under this pressure, the height of DEAE-cellulose in the column was from 28 to 30 cm. The column was enclosed in a water jacket, which was maintained at 25° by means of a circulating water pump. A column of fresh DEAE-cellulose was prepared for each experiment.

After equilibration was complete, several fractions of the effluent were collected to serve as a blank for the optical density measurements. The sample to be chromatographed was applied to the top of the column, and allowed to drain into the DEAE-cellulose at the fastest rate possible (approximately 1 ml. per minute). The column was then connected to a constant pressure reservoir containing pH 5.5, 0.10 M acetate buffer, and eluted with the buffer until the absorption of the effluent

dropped to the reading of the blank. A double gradient of increasing NaCl concentration and decreasing pH was obtained by running pH 3.6, 0.10 M acetate buffer, containing 0.25 M NaCl into a 300 ml. mixing vessel filled with the starting buffer. The mixing vessel was stirred continuously with a magnetic stirrer. This gradient was used to elute the remaining absorbed material from the column. The flow rate for all experiments reported was from 0.6 to 0.8 ml. per minute. A complete description of the gradient changes occurring in the eluent solution is given in Table X. The theoretical pH and chloride ion concentrations were calculated from the formula of Alm, et al (1), and were found to agree precisely with measurements.

The chromatographic effluent was fractionated into 11 ml. fractions by means of an automatic fraction collector. The absorption of each fraction was read at 280 mu in a Beckman DU spectrophotometer, using a 0.20 mm. slit width. An elution diagram was constructed by plotting the optical density of each fraction versus the fraction number. The chromatographic pattern thus obtained was evaluated by measuring the area under the absorption peaks with a planimeter.

In order to facilitate comparison of the various effects of urea and alkali on pepsin, all chromatograms reported in this investigation were technically identical. In this way, the differences noted are solely due to the treatment of the

TABLE X

GRADIENT ELUTION SYSTEM FOR THE CHROMATOGRAPHY OF PEPSIN*

FRACTION[a] NUMBER	V_g	THEORETICAL[b]		MEASURED[c]	
		Cl^-	pH	Cl^-	pH
0-20	0 ml	0	5.50	0	5.50
34	154	0.086 M	4.85	0.085 M	4.85
47	297	0.142	4.42	0.137	4.47
61	451	0.178	4.15	0.172	4.18
75	605	0.203	3.96	0.199	3.99
88	748	0.219	3.84	0.217	3.87
102	902	0.230	3.76	0.224	3.80

*Starting buffer : pH 5.5, 0.10 M acetate
Gradient buffer : pH 3.6, 0.10 M acetate, 0.25 M NaCl

a) Each fraction = 11 ml. The gradient buffer was started at Fraction #20.

b) Theoretical calculations were obtained with the use of the equation (1):

$$C/C_o = (e^K - 1) / e^K$$

C = Cl^- concentration of effluent; pH of starting buffer minus pH of effluent.
C_o = Cl^- concentration of gradient buffer; pH of starting buffer minus pH of gradient buffer.
$K = V_g / V_s$
V_s = Volume of starting buffer in mixing vessel = 300 ml.
V_g = Volume of gradient buffer added to mixing vessel.

c) Chloride concentration was measured by titration with $AgNO_3$ in the presence of K_2CrO_4. pH was measured with a Beckman Model G pH meter.

pepsin sample prior to chromatography. However, the fraction number at which gradient elution was started in each chromatogram varied throughout all experiments from about Fraction #15 to #30. As will be seen from the results, this variation in no way affected the subsequent developement of the absorbed protein material, once the gradient buffer was applied. The position (or fraction number) of each absorption peak was effectively measured as the difference between the fraction in which the absorption peak appeared and the fraction at which the gradient was applied. Therefore, all the chromatographic elution patterns reported in this investigation have been corrected so that gradient elution has been started at Fraction #20. This eliminates the need for speaking in terms of difference fractions, and allows visual comparison of the figures presented.

B. Estimation of Protein

The absorption of protein solutions at 280 mμ is essentially due to the tyrosine and tryptophane content of the protein (7). In the present investigation, the breakdown products of pepsin were detected by the 280 mμ absorption of the effluent fractions. In order to be certain that all of the breakdown products could be detected by this method, it was essential to test the effluent fractions by another method which was not dependent on tyrosine and tryptophane. Two such methods

were chosen: the ninhydrin reaction for α-amino groups, and Nessler's reaction for nitrogen. Although the quantitative results were different, both methods indicated a qualitative distribution of protein material identical to that found by absorption at 280 mμ.

Since all the breakdown products absorb at 280 mμ, the measurement of the optical density of the chromatogram fractions is the simplest, most convenient and reliable method. The determination of absolute protein concentrations of the breakdown products of pepsin by light absorption presupposes that all the products arising from pepsin have the same tyrosine and tryptophane contents. All products would then have the same extinction coefficient as pepsin. This could hardly be expected to be the case. However, the determination of relative concentrations of the breakdown products is possible since the absorption of a solution of native pepsin is essentially the same as a solution of pepsin breakdown products, on a weight basis. This is demonstrated by Table XI, which lists the extinction coefficient of pepsin in various solutions. The extinction coefficient of pepsin decreases only three per cent after autolysis for 100 hours at pH 1.8 and pH 5.3. There is even less change after alkaline inactivation of pepsin at pH 6.9.

As stated previously, the area under the curves of the elution patterns obtained by plotting the optical density (O.D.)

TABLE XI

EXTINCTION COEFFICIENT OF PEPSIN IN VARIOUS SOLUTIONS

Pepsin Solution	$E^{0.1\%}_{1\ cm.}$ (280 mu)
pH 1.8	1.53
pH 1.8 - 100 hours	1.48
pH 5.3	1.49
pH 5.3 - 100 hours	1.45
pH 6.9 - 2 hours	1.53

of the fraction against the fraction number was measured by planimetry. The same size of coordinate paper was used for all measurements, and the planimeter was calibrated regularly. The recovery of total protein was based on the total area under the elution diagram. Table XII shows the planimeter measurements of the total curve area for some of the experiments. These measurements were used as an indication of the recovery of protein from the chromatographic procedure. It is evident that there is no drastic loss of total protein absorption under any of the conditions reported in Table XII, regardless of the extent of loss of active pepsin (last column). The average total area per 50 mg. of pepsin sample for all the curves is 44.5 ± 1.7 cm^2. This total area depends on the size of the scale used for plotting

TABLE XII

RECOVERY OF PROTEIN FROM CHROMATOGRAPHY

AREA UNDER OPTICIAL DENSITY VS. FRACTION NUMBER CURVES

Expt. No.	Pepsin Treatment	Total area per 50 mg.	D*	% Pepsin Remaining‡
143	pH 5.5, "0" Min.	45.7 $cm.^2$	+1.2	100
148	pH 5.5, 37°, 60 Min.	46.4	+1.9	94.4
176	pH 3.5, 37°, 200 Min.	41.4	-3.1	74.0
	Average (44.5)		(±2.1)	
	8 M urea, 37°			
164	pH 5.5, 1 Min.	42.6	-1.9	90.6
153	pH 5.5, 15 Min.	45.2	+0.9	53.4
157	pH 5.5, 45 Min.	44.3	-0.2	34.6
179A	pH 5.5, 155 Min.	49.1	+4.6	11.5
175	pH 2.5, 30 Min.	44.8	+0.3	19.9
174	pH 4.0, 30 Min.	46.1	+1.6	43.4
	Average (45.4)		(±1.6)	
167	pH 6.7, 23°, 15 Min.	42.8	-1.7	55.4
172A	pH 6.7, 23°, 90 Min.	41.9	-2.6	23.2
177	pH 7.0, 23°, 15 Min.	43.8	-0.7	3.2
	Average (42.8)		(±1.7)	
	Total Average (44.5)		(±1.7)	

*D - deviation from total average.

‡ - Chromatographic recovery of the enzymatically active pepsin component. The pepsin recovered in experiment #143 was taken as 100%.

the effluent diagrams. The scale was 0.150 O.D. - fraction units per square centimeter of paper. Since each fraction represented 11 ml. volume of effluent, the scale was 1.65 O.D. - ml. units per square centimeter of paper. The average total curve area (per 50 mg. of pepsin) of 44.5 cm^2 then represents:

$$\frac{44.5\ cm^2}{50\ mg.} \times 1.65\ O.D.\text{-}ml./cm^2 = 1.47\ O.D./mg./ml.$$

The value of 1.47 then represents the extinction coefficient of a 0.10 per cent pepsin solution, and it is found to agree with the values of extinction reported in Table XI.

C. Determination of Proteolytic Activity

All assays for peptic activity were based on the method of Northrup, Kunitz, and Herriott (56), using hemoglobin as the substrate. Hemoglobin solution was prepared fresh, immediately before use by dissolving 2.0 gm. hemoglobin powder (Worthington Biochemical Corp.) in 70 ml. of distilled water, then adding 20.0 ml. of 0.30 N HCl. The mixture was then diluted to 100 ml. with distilled water. The final pH of the substrate solution was 1.6.

For the assay, 5.0 ml. of hemoglobin solution was placed in a water bath at 35.5° for ten minutes, then 1.0 ml.

of the solution to be assayed, previously diluted to the proper enzyme concentration, was added. After ten minutes, 10.0 ml. of 0.3 M trichloroacetic acid (TCA) was added to stop the reaction. The tube was mixed well, allowed to stand about 15 minutes, and the contents were filtered through Whatman #41 filter paper. The TCA filtrate was then diluted 1:3 with water, and the optical density of this solution was read at 280 mu in a Beckman DU spectrophotometer. The filtrates were read against a hemoglobin blank solution treated in a manner identical to that of the samples, except that water was used in place of the enzyme solution. The blank had an optical density of about 0.110 at 280 mu, when read against water.

The digestion of 2.0 per cent hemoglobin solution by pepsin at pH 1.6 is shown in Table XIII. A solution of pepsin (0.22 mg./ml.) was diluted to give the pepsin concentrations listed in the first column of the table. The hydrolysis appears to be linear up to a pepsin concentration of 0.044 mg./ml. This concentration corresponds to a 280 mu absorption of about 0.066 optical density units. On this basis, all chromatographic fractions that were known to have peptic activity, were diluted with water before assaying, so that they would have an optical density reading of less than 0.06 at 280 mu. Fractions which were suspected of having no activity were assayed without dilution, and in most cases, the assay time for these fractions was extended

TABLE XIII

DIGESTION OF HEMOGLOBIN SOLUTION BY PEPSIN AT pH 1.6 [a]

Pepsin Concentration [b] (mg/ml)	Folin Reaction [c] of TCA Filtrate (Klett units)	Optical Density [d] of TCA Filtrate (280 mu)
0.0022	10	0.030
0.0044	21	0.046
0.0088	53	0.107
0.022	130	0.275
0.044	260	0.555
0.073	308	0.671
0.22	423	0.907

a) Digestion mixture: 5.0 ml of 2.0% hemoglobin solution, (pH 1.6) and 1.0 ml enzyme solution, at 35.5° for ten minutes. Reaction stopped by addition of 10.0 ml 0.3 M trichloroacetic acid (TCA).

b) Dilutions of a 0.22 mg/ml solution of pepsin.

c) 1.0 ml of TCA filtrate + 3.0 ml 1.5 M Na_2CO_3 + 1.0 ml Folin reagent + 5.0 ml distilled water. Klett #66 filter. (Standard: 0.001 meq. tyrosine = 704 Klett units per 10.0 ml volume)

d) Beckman D U optical density readings of TCA filtrate diluted 1 : 3 with distilled water, against Hb blank solution. (56)

to 20 or 30 minutes. When a chromatogram was assayed for proteolytic activity, every other fraction in the vicinity of the absorption peaks was tested.

One unit of pepsin activity was originally defined as "that quantity of pepsin which would produce one milliequivalent of tyrosine (measured by Folin's phenol reagent), not precipitated by 0.3 M trichloroacetic acid, per minute per 6.0 ml. standard hemoglobin digestion mixture, at 35.5°" (3). The tyrosine equivalent of a solution as determined by Folin's reagent differs from that obtained by ultraviolet absorption, due to the fact that tyrosine and tryptophane affect the two measurements differently. In order to obtain an absolute value for pepsin units, it was necessary to carry out a series of determinations with a single pepsin solution using the Folin's phenol reagent method. These results are shown in column two of Table XIII. The activity of the pepsin sample, calculated on the basis of these measurements is about 0.10 $\left[\text{P.U.}\right]^{\text{Hb}}_{\text{mg N}}$

The calculation of original pepsin units was considered to be too cumbersome for use in the present investigation. Instead, the relative peptic activity of the chromatographic fractions was calculated as the total increase in the 280 mu absorption of the standard hemoglobin TCA filtrate (i.e. 6 ml. hemoglobin digest + 10 ml. TCA) caused by 1.0 ml. of the enzyme fraction, divided by the absorption of the fraction. The

calculation is then simplified as follows:

$$\frac{(O.D.)_{Hb} \times D_{Hb} \times D_F}{(O.D.)_F} = \text{Relative Pepsin Units}$$

where: $(O.D.)_{Hb}$ = optical density difference of the digested hemoglobin TCA filtrate at 280 mu.

$(O.D.)_F$ = optical density of the fraction at 280 mu.

D_F = dilution factor of the fraction (if any).

D_{Hb} = dilution factor of the hemoglobin TCA filtrate (this factor is 3 in the present method).

The relative specific activity of the pepsin sample used for these experiments is 25.9. To calculate the relative specific activity of pepsin on a weight basis, this expression for pepsin activity must be multiplied by the extinction coefficient (280 mu) of the sample. Pepsin has an extinction of 1.48 O.D. units/mg./ml.; therefore, the relative specific activity per mg. of pepsin is 37.3.

D. Treatment of Pepsin Prior to Chromatography

Pepsin was treated with solutions of varying pH and composition. The actual treatment prior to chromatography depended on the compositon of the solvent. All experiments were

performed on 49 to 51 mg. of the pepsin preparation. The samples were dissolved in the various solvents at the temperature indicated for the experiment. At the end of the incubation period, all solutions were adjusted to pH 5.5, and applied to the DEAE-cellulose column as rapidly as possible. The incubation period of pretreatment was from the time of the addition of the solvent to the pepsin, to the time of adjustment of the solution to pH 5.5. The pH adjustment was not necessary for all experiments, since some of the pretreatments were carried out at pH 5.5. In this case, the incubation period indicates the time up to the application of the solution to the column. It was necessary to time the incubation period in this way, because the time required for the sample to enter the column of DEAE-cellulose was found to vary between five and ten minutes.

In some experiments, the protein was found to cause a sharp change in the pH of the buffer solution. The pH values reported for all experiments were those of the buffer solution in the presence of the protein, as measured with a Beckman model "G" pH meter. No corrections were made for the influence of urea on the pH values. The pH meter was always calibrated with standard buffers both before and after the pH of a sample was measured.

Old solutions of urea are known to contain small amounts of cyanate. Under moderate conditions, cyanate can

react slowly with the amino groups of proteins to yield carbamyl derivatives (81a). In order to avoid possible reactions due to cyanate, the urea solutions were made fresh immediately prior to use. As a further precaution, the urea solutions were first acidified with acetic acid and allowed to stand about 15 minutes before addition of the proper amount of base to give the desired pH. Acidification of urea solution decomposes any cyanate present.

The urea present in the pepsin samples was not adsorbed to DEAE-cellulose. It was washed through the column with the first 60 ml. of the eluent buffer. The 280 mu molar extinction coefficient of urea is about 0.016. Therefore, the increase in optical density of the effluent caused by urea was considered to be negligible.

In all of the experiments except those in which phosphate buffer was used, 50 mg. of pepsin was dissolved in 5.0 ml. of solvent. In experiments using phosphate buffer, pH 6.7 and 7.0, 2.0 ml. of buffer was added to 50 mg. of the pepsin preparation. At the end of the incubation period, the solutions were acidified to pH 5.5 and diluted to a final volume of five ml. All of the experiments in aqueous buffer at pH 6.7 and 7.0 were performed at 23°, in order to obtain a slow rate of pepsin inactivation.

E. Supplemental Experiments

Dialysis experiments were performed on urea inactivated and alkaline inactivated pepsin samples, to obtain an indication of the size of the breakdown products of pepsin (Experiments #179 and #172). After treatment of the pepsin, one-half of the sample (5.0 ml.) was chromatographed immediately. The other half was dialysed against pH 5.5, 0.1 M acetate buffer for 24 to 26 hours at 6° before chromatography.

The 280 mu extinction of tryptophane is about four to five times greater than that of tyrosine (7, 95). In the Folin's phenol reaction the color yield of tryptophane is only 90 per cent that of tyrosine. Because of this difference, the analysis of the chromatographic fractions by both methods should give an indication of the similarity or dissimilarity of the fragments from pepsin inactivation.

The fractions from two chromatograms were analysed by the Folin's phenol reaction (Experiments #158 and #169). Three milliliters of each fraction were added to 2.0 ml. of 1.5 M Na_2CO_3, followed by the addition of Folin's phenol reagent. After 30 minutes, the color density of each tube was read on a Klett-Summerson colorimeter, using a #66 filter. The final pH of the reaction mixture to which Fraction #1 was added was 10.1; that of Fraction #100 was 9.8. This slight difference in pH had

no effect on the total color developement. For convenience, the Klett readings of the reaction mixtures were converted to optical density units, and plotted on the same scale as the optical density of the fractions (Fig. 9).

In one experiment, the solubility of the products of inactivation in ten per cent TCA was tested (Experiment #177). One milliliter of 60 per cent TCA was added to 5.0 ml. of each chromatographic fraction. The mixture was warmed at 80° for 15 minutes, cooled to room temperature, and then centrifuged for 20 minutes. The supernatent was poured into one centimeter cuvettes and read in a Beckman DU spectrophotometer at 280 mμ, against a blank solution of ten per cent TCA. The optical density readings were corrected for dilution and plotted on the same optical density scale as the untreated fractions for comparison.

Several components from the chromatograms were analysed for N-terminal amino acids (46, 58, 80). The fractions containing the component were pooled and lyophilized. The dry residue was then redissolved in 10 ml. of two per cent $NaHCO_3$ and 1 ml. of an acetone solution containing 5 mg. dinitrofluorobenzene (DNFB) was added. After three or four hours, the solution was extracted with 5 X 5 ml. portions of ether to

extract excess DNFB, and 6 ml. of concentrated HCl were added. The mixture was boiled down to a volume less than 10 ml., and then refluxed for 20 hours. The dinitrophenyl-(DNP)-amino acids were extracted from the acid solution with ether. The ether was dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. Excess dinitrophenol was removed from the ether residue by sublimation. The DNP amino acids were identified by paper chromatography in a system using n-amyl alcohol saturated with 0.05 M phthalate buffer, pH 6.0 (59).

CHAPTER IV

EXPERIMENTAL OBSERVATIONS

The chromatographic pattern obtained from the untreated pepsin sample is shown by the solid line in Fig. 1. Fifty milligrams of pepsin were dissolved in 5.0 ml. of 0.10 M acetate buffer, pH 5.5, and applied to the column immediately. The chromatogram was first developed with 0.10 M acetate buffer, pH 5.5, until the optical density of the effluent returned to the optical density of the blank solution. A gradient of increasing chloride ion concentration and decreasing pH was started at Fraction #20 (Table X). This gradient was found to elute all adsorbed protein from the DEAE-cellulose.

The chromatogram shown in Fig. 1 was divided into three areas: "A", "B" and "P". All protein material eluted before starting the gradient has been designated as "A". The symbol "P" has been used to designate the protein of the major component, which comprises about 70 per cent of the total material in the chromatogram. The remaining absorbing material, that which appears after the gradient was started and before the "P" component was eluted, has been assigned the symbol "B".

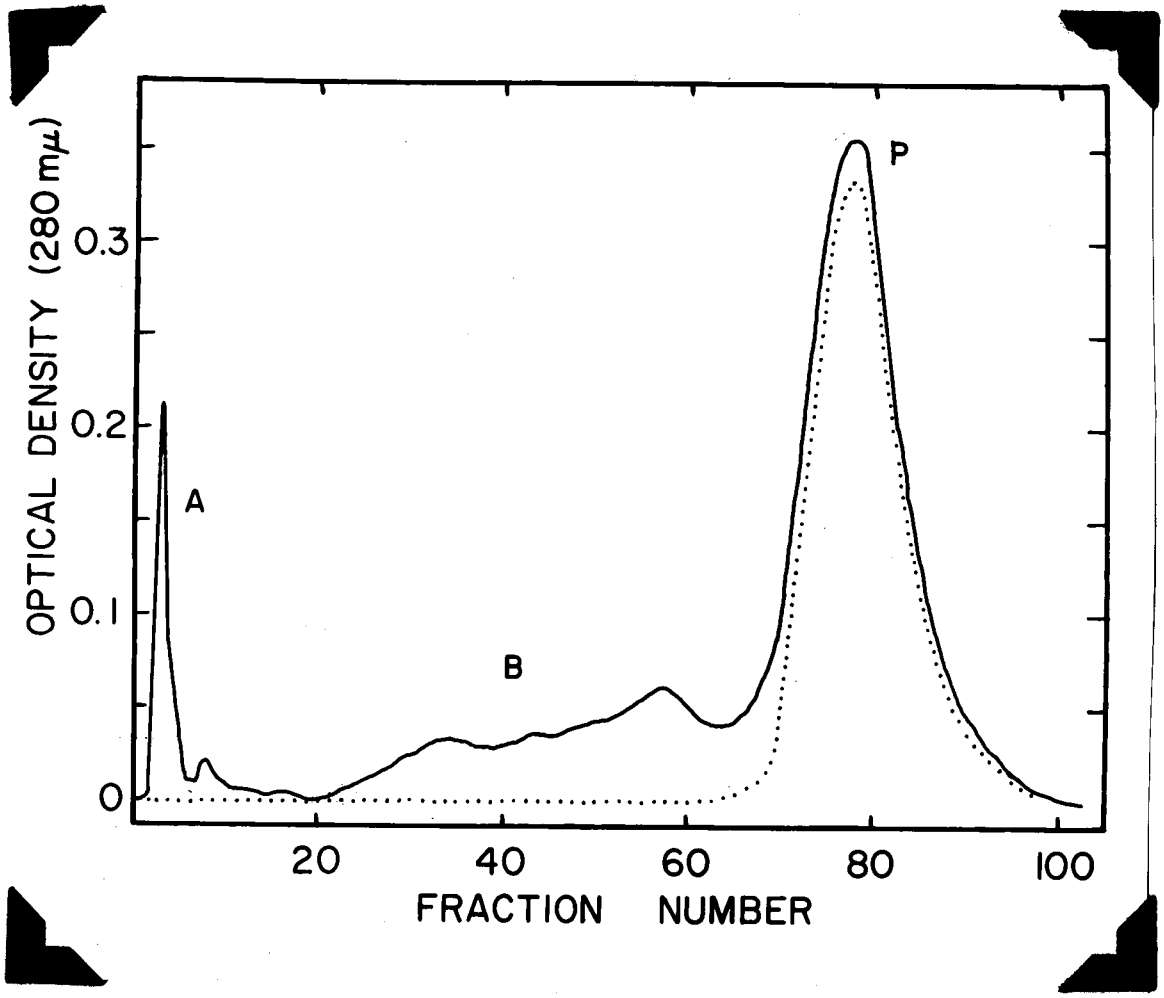


FIG. 1

THE CHROMATOGRAPHY OF PEPSIN ON DEAE-CELLULOSE

Experiment #143: Solid line - optical density of the fractions from a chromatogram of 50 mg. of pepsin dissolved in 5.0 ml. 0.10 M acetate buffer, pH 5.5. Each fraction is 11 ml. Dotted line - enzymatic activity toward hemoglobin shown as the increase in optical density of a hemoglobin TCA filtrate (diluted 1:30) after treatment with 1.0 ml. of the fraction.

The dividing line between the "P" component, and the "B" component was obtained by extending a line down the ascending side of the "P" component to the baseline. The "B" component is undoubtedly heterogeneous, but there is little distinction upon which to base a clear division into subcomponents. It will be seen later that the changes occurring in this area of the chromatogram, upon treatment of the sample with urea or alkali, permit further subdivision.

The major peak of component "A" of the chromatogram is unusual in that it is not retarded by the DEAE-cellulose, under the conditions used for chromatography. This might indicate that "A" consists mainly of small peptides, or material having a low net negative charge. In contrast, the protein of components "B" and "P" are still firmly adsorbed to the ion-exchanger when the concentration of acetate buffer is raised to 1.5 M, at pH 5.5, or when the pH of the 1.5 M eluent is dropped to 4.0. An ion with a higher electronegativity than acetate, such as Cl^-, is necessary to elute components "B" and "P" from the column.

The dotted line in Fig. 1 is a plot of the enzymatic activity of the various chromatographic fractions toward hemoglobin. It can be seen that only the "P" component has peptic activity. The relative specific activity of this component is 26.9, as compared to a relative specific activity of 25.9 of the pepsin preparation before chromatography. This slight increase

in specific activity may or may not be significant, but on the basis of the chromatographic pattern, it should be much higher. Since only 70 per cent of the total absorbing material has activity, and the relative specific activity measurement is based on the absorbing material present in the sample, it appears likely that some pepsin has been inactivated by the procedure.

A. The Autodigestion of Pepsin

When pepsin is dissolved in 0.10 M acetate buffer, pH 5.5, and incubated at 37° for one hour, only slight differences in the chromatographic pattern from that shown in Fig. 1 can be noticed. The "P" component is found to decrease about five per cent, with a corresponding increase in absorption in the "A" and "B" components. Most of the increase takes place in the "A" component. This loss of "P" increases with increasing length of the incubation period. If the fractions containing "P" are pooled, lyophilized, and then rechromatographed, a chromatographic pattern that is qualitatively identical to that in Fig. 1 is obtained. The recovery of "P" will depend on the time lapse between chromatography and rechromatography.

This only demonstrates what is already well known about pepsin; i.e., that pepsin will digest itself in acid media, even at pH 5.5. In order to demonstrate the autolysis of pepsin more clearly, 50 mg. of the preparation was dissolved in 5.0 ml.

of HCl-NaCl solution at pH 3.5, and allowed to stand at 37° for 200 minutes before chromatography. The result is shown in Fig. 2. Qualitatively, the pattern appears similar to that in Fig. 1, except for the disappearance of the small peak at Fraction #59. The "P" fraction has decreased 26 per cent. A corresponding increase appears in the "A" component, while the "B" component has decreased slightly. These changes in the chromatographic distribution of protein are recorded in Table XIV, along with the distribution found in the chromatographic pattern shown in Fig. 1 (Experiment #143 is shown in Fig. 1; Experiment #176 is shown in Fig. 2).

B. The Effect of 8 M Urea

When pepsin is incubated in 8 M urea-acetate buffers at pH 5.5, 37°, there is a rapid decrease in the "P" component of the chromatographic pattern, with corresponding increases in both the "A" and "B" components. Only 53 per cent of the "P" component remains after 15 minutes incubation. These results are described in Fig. 3 and Table XV.

Fig. 3 shows that gross changes are occurring in both the "A" and the "B" components. These changes are quite different from those caused by autolysis in aqueous solution (Fig. 2). In component "A", a second, strong absorption peak appears at Fraction #7. Component "B" resolves into at least six components,

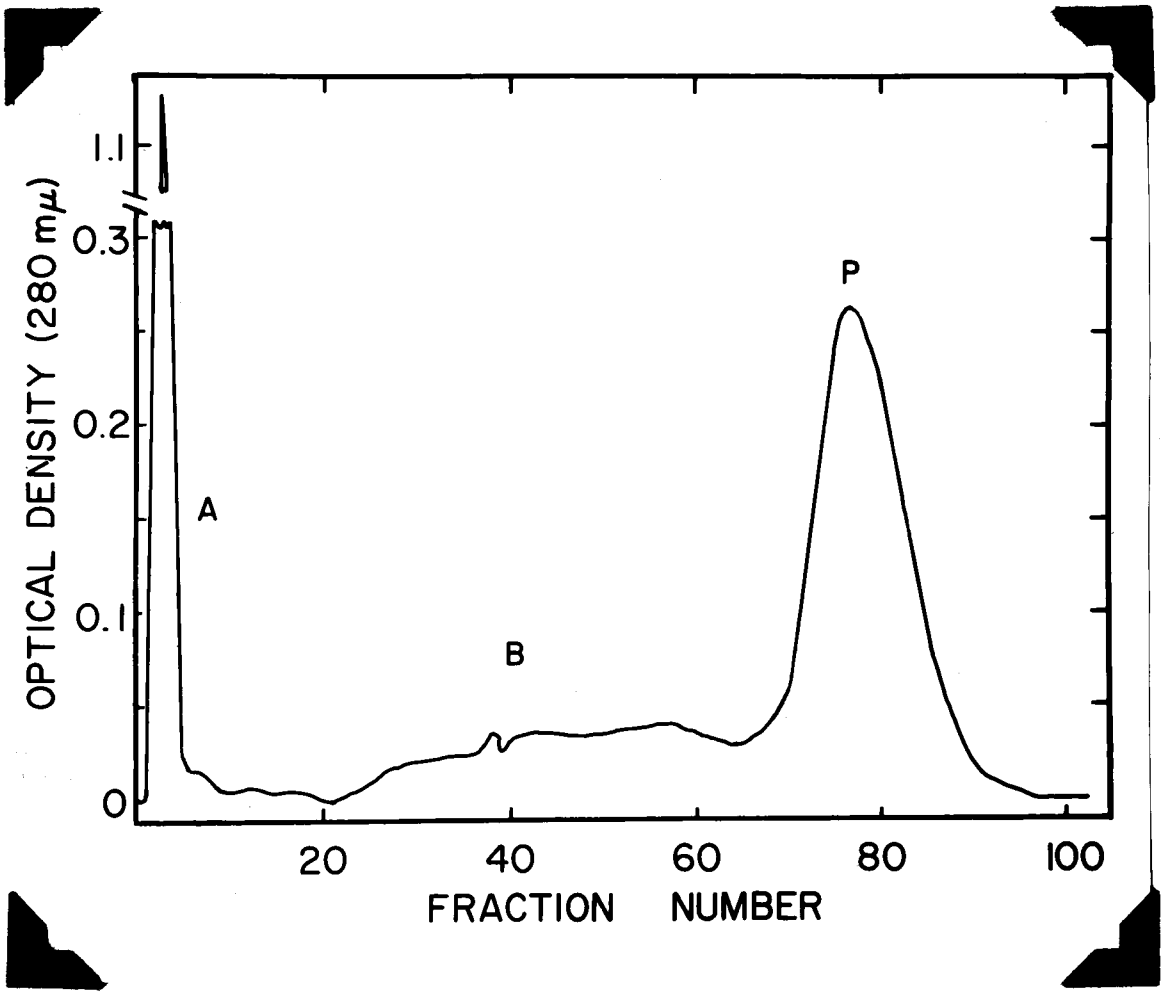


FIG. 2

THE AUTODIGESTION OF PEPSIN

Experiment #176: Optical density of the fractions from a chromatogram of a pepsin sample incubated at pH 3.5, 37°, for 200 minutes.

TABLE XIV

THE CHROMATOGRAPHIC DISTRIBUTION OF THE AUTOLYSIS PRODUCTS OF PEPSIN

Expt. No.	Conditions*	% Total Absorption "A"	"B"	"P"	Pepsin**
143	pH 5.5, 1 Min., 23°	7.0%	23.6%	69.4%	100 %
148	pH 5.5, 60 Min., 37°	10.1	24.4	65.5	94.4
176	pH 3.5, 200 Min., 37°	27.8	20.9	51.3	74.0

*1.0 % pepsin solutions

pH 5.5 - 0.1 M acetate buffer, ionic strength = 0.09

pH 3.5 - HCl - NaCl, ionic strength = 0.10

**The pepsin ("P") component of Experiment #143 is taken as 100%.

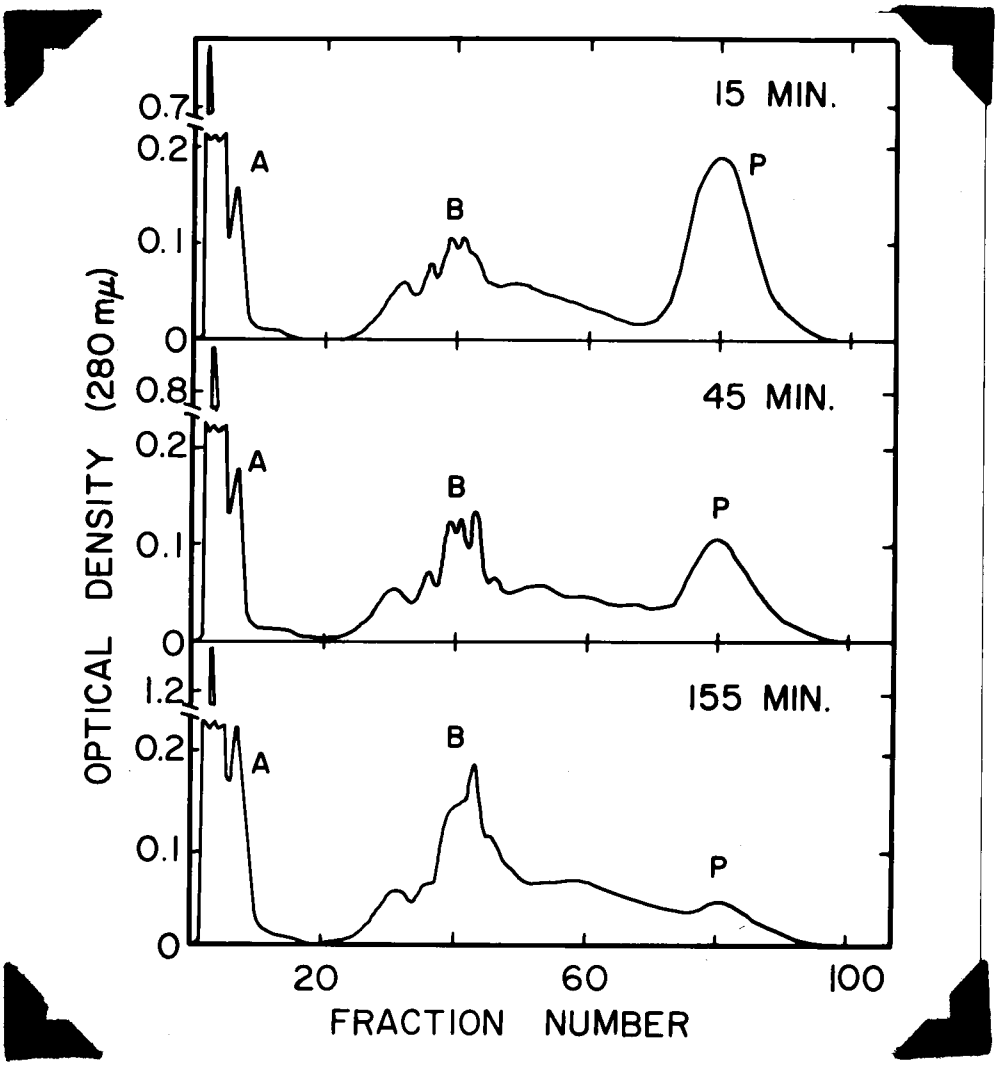


FIG. 3

THE CHROMATOGRAPHY OF UREA INACTIVATED PEPSIN

Experiment #153, #157, #179A: 50 mg. pepsin in 5.0 ml. 8 M uren-acetate at pH 5.5, 37°, for the indicated length of time.

TABLE XV

EFFECT OF INCUBATION IN 8 M UREA ON THE CHROMATOGRAPHIC PATTERN OF PEPSIN*

Expt. No.	Incubation Time	% Total Absorption			Pepsin**
		"A"	"B"	"P"	
143	(no urea)	7.0%	23.6%	69.4%	100 %
164	1 Min.	12.0	25.2	62.8	90.6
160	5 Min.	16.1	31.7	52.2	75.3
153	15 Min.	26.3	36.7	37.0	53.4
152	30 Min.	32.4	37.6	30.0	43.3
157	45 Min.	33.6	42.4	24.0	34.6
158	60 Min.	38.1	43.0	18.9	27.3
179A	155 Min.	46.5	45.5	8.0	11.5

*1.0% pepsin solutions in 8 M urea - 0.10 M acetate buffers at pH 5.5, 37°.

**The pepsin ("P") component of Experiment #143 is taken as 100%.

all of which are eluted within less than 20 fractions. The absorption peaks can be most easily distinguished in the 30, 45, and 60 minute chromatograms (see Fig. 9A). After incubation of the sample for 155 minutes, the increase in absorption of Fraction #43 has overshadowed the absorption peaks in Fractions #39 and #41.

The data of per cent total absorption from Table XV has been plotted in Fig. 4. This plot shows that the loss in the "P" component is paralleled by approximately equal formation of protein in the "A" and "B" components.

The effect of pH on the urea inactivation of pepsin was studied by incubating pepsin in 8 M urea solutions at pH 2.5, 4.0, 5.5 and 5.8, for 30 minutes at 37°. Fig. 5 illustrates that distinct differences appear in the chromatograms of the pepsin solutions incubated at pH 5.5, 4.0 and 2.5. A new peak is found to appear in Fraction #59 of the chromatograms of pepsin incubated at pH 5.8, 4.0 and 2.5 in 8 M urea solution. The absorption of the peaks in Fractions #30 to #45 is less when the pepsin is incubated at pH 4.0 or 2.5, than when incubated at pH 5.5 or 5.8. In order to measure the distribution of protein in these chromatograms, component "B" was divided into two areas. The area under the new peak at Fraction #59 has been labeled "C", while "B" now indicates the protein eluted prior to "C".

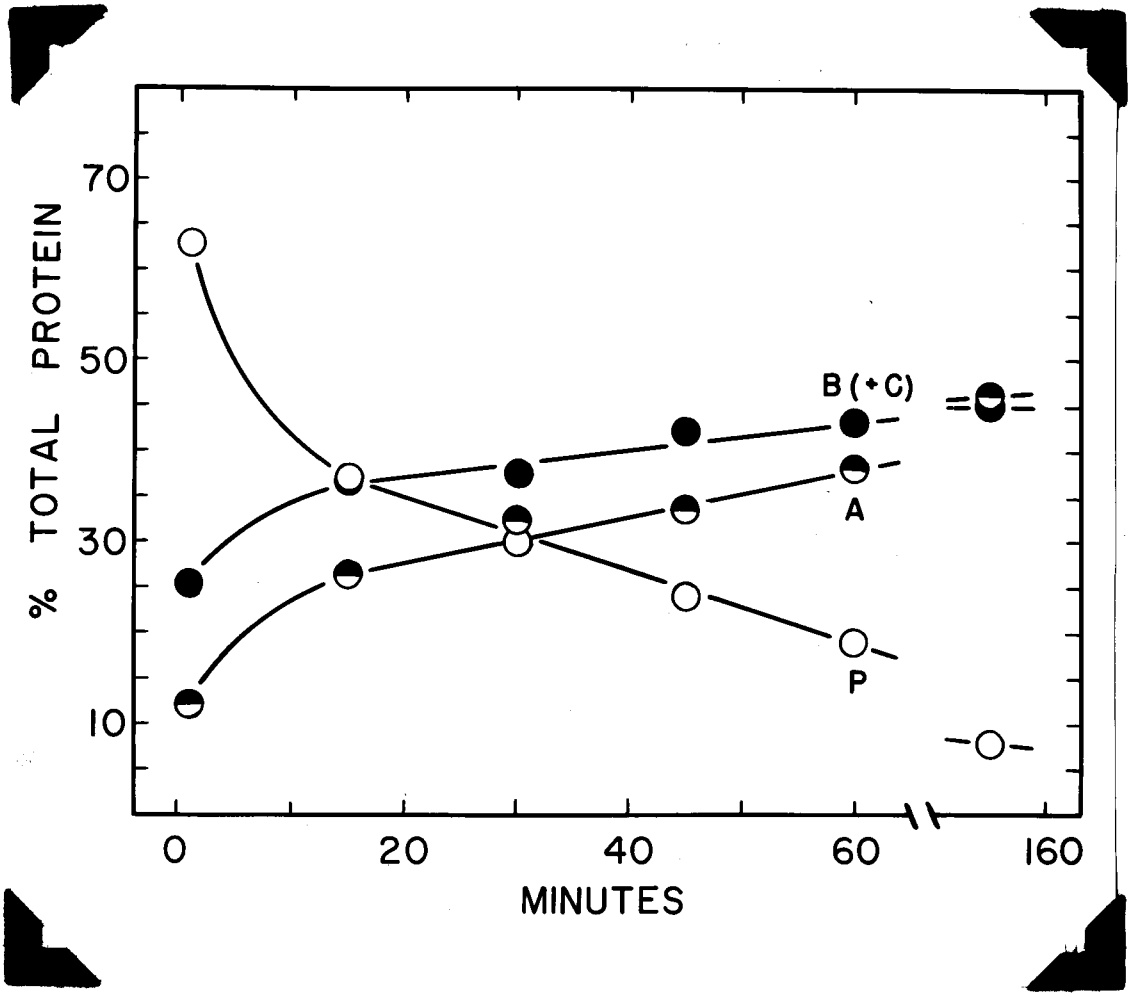


FIG. 4

THE INACTIVATION OF PEPSIN IN 8 M UREA AND THE FORMATION OF NON-PEPSIN PROTEIN MATERIAL

Pepsin was incubated in 8 M urea-acetate at pH 5.5, 37°, for the indicated time prior to chromatography. Components were measured by planimetry of the area under the chromatographic elution patterns. (see Table XV)

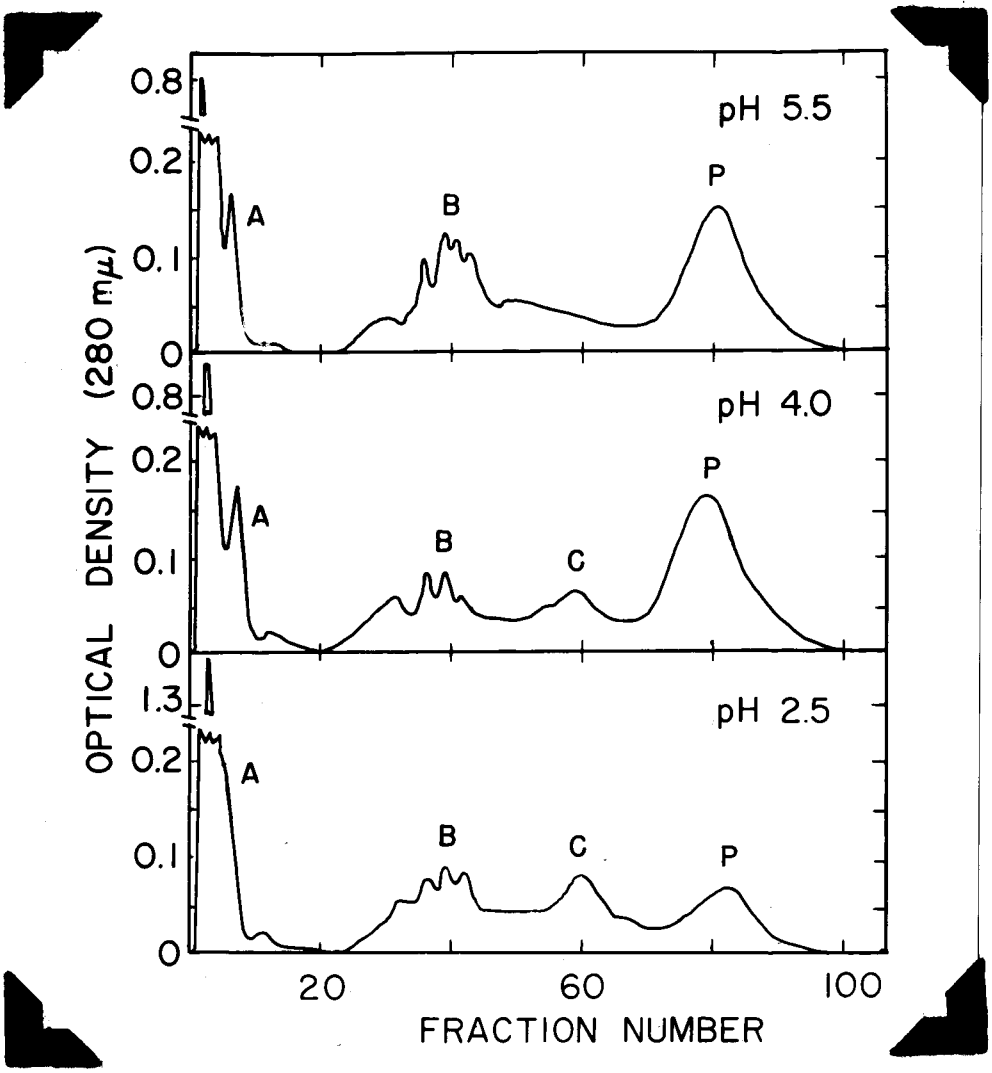


FIG. 5

THE EFFECT OF pH ON THE INACTIVATION OF PEPSIN IN 8 M UREA SOLUTION

Experiments #152, #174, #175: Pepsin was incubated at the indicated pH in 8 M urea, 37°, for 30 minutes prior to chromatography.

In order to divide and measure both areas, an arbitrary line has been drawn from the optical density reading of Fraction # 55 to the baseline at Fraction # 50. This line approximately corresponds to the peak in component "C".

The per cent total absorption of components "A", "B", "C" and "P" are given in Table XVI. Since component "C" is not present in the chromatogram of the pepsin sample treated at pH 5.5, the sum of "B+C" components have been included. "B+C" corresponds to the area measured in Table XV as "B". The "C" component of the data reported in Table XV comprised from 11 to 17 per cent of the total absorption, depending on the length of the incubation period. The data from Table XVI has been plotted in Fig. 6.

In 8 M urea solutions, the "P" component is present in approximately the same amount after incubation at pH 4.0 and pH 5.5, and decreases to 13.2 per cent when the pepsin is incubated at pH 2.5, and to 8.4 per cent when incubated at pH 5.8, for the same length of time. The opposite is noted for components "B" and "C", which are produced minimally at pH 4.0 and pH 5.5, respectively. Since the formation of "B" and "C" is dependent on the total loss of "P", it would appear that the total of both components, "B+C", is actually less at pH 2.5 than it is at pH 4.0. The formation of component "A" in 8 M urea solution increases consistantly with decreasing pH, even if the

TABLE XVI

THE EFFECT OF pH ON THE INACTIVATION OF PEPSIN IN 8 M UREA SOLUTIONS*

Expt. No.	pH	% Total Absorption				
		"A"	"B"	"C"	"B+C"	"P"
175	2.5[a]	48.2%	22.6%	16.0%	38.6%	13.2%
174	4.0[b]	40.7	17.8	12.7	30.5	28.8
152	5.5[b]	32.4	26.5	11.1	37.6	30.0
150	5.8[b]	21.5	38.8	31.3	70.1	8.4

*1.0 % pepsin in 8 M urea solutions at 37° for 30 minutes prior to chromatography.

a) 0.10 M acetate - HCl

b) 0.10 M acetate buffers

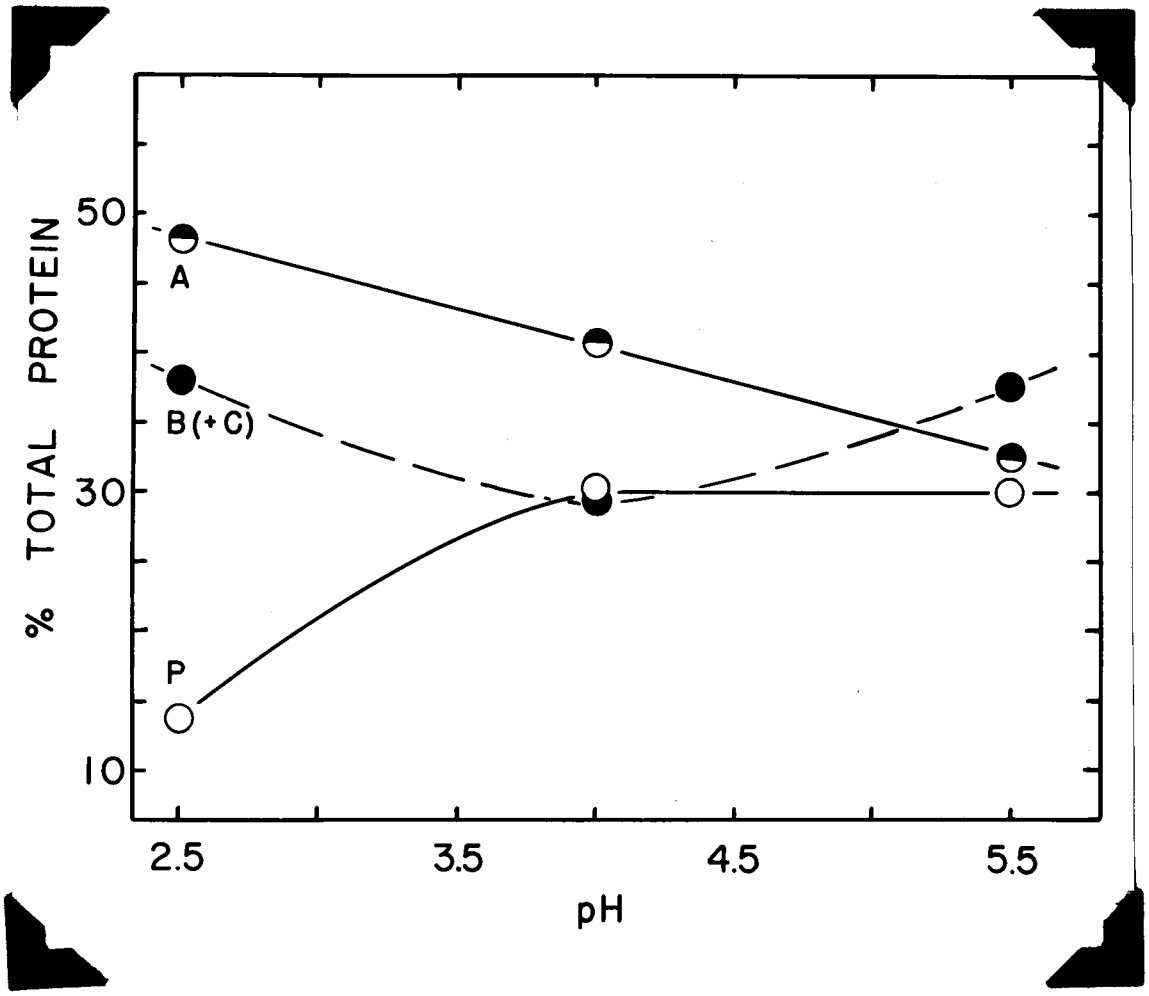


FIG. 6

THE EFFECT OF pH ON THE INACTIVATION OF PEPSIN IN 8 M UREA AND THE FORMATION OF NON-PEPSIN PROTEIN MATERIAL

Pepsin was incubated in 8 M urea at 37° for 30 minutes prior to chromatography. Components were measured by planimetry of the area under the chromatographic elution patterns. (see Fig. 5 and Table XVI)

total breakdown of the "P" component is taken into consideration. It would appear, then, that as the pH of the 8 M urea solution is decreased, the "B+C" components are formed in decreasing amounts, while the "A" component is formed in increasing amounts. It should be noted that the ionic strength of the buffers used in these experiments were not the same. This may account for some of the differences noted.

C. The Effect of Alkaline Inactivation

In order to establish the effect of alkaline inactivation on the chromatography of pepsin, the enzyme was incubated in aqueous buffer at pH 6.7, 23°, for 15, 45 and 90 minute periods prior to chromatography. Fifty milligrams of the pepsin preparation was dissolved in 2.0 ml. of pH 7.0, 0.10 M phosphate buffer at 23°. The pH of the buffer decreased to 6.7 immediately upon addition of the protein and remained constant thereafter throughout the incubation period. When the protein was dissolved in 0.10 M acetate buffer at pH 5.5, either in aqueous buffer or in the presence of urea, no change in pH was observed.

The effect of incubation of pepsin at pH 6.7 is shown in Fig. 7. The rate of loss of the "P" component is similar to that found when the pepsin is dissolved in 8 M urea at pH 5.5, 37°. However, the chromatographic patterns obtained from the two treatments are markedly different. At pH 6.7, the loss of

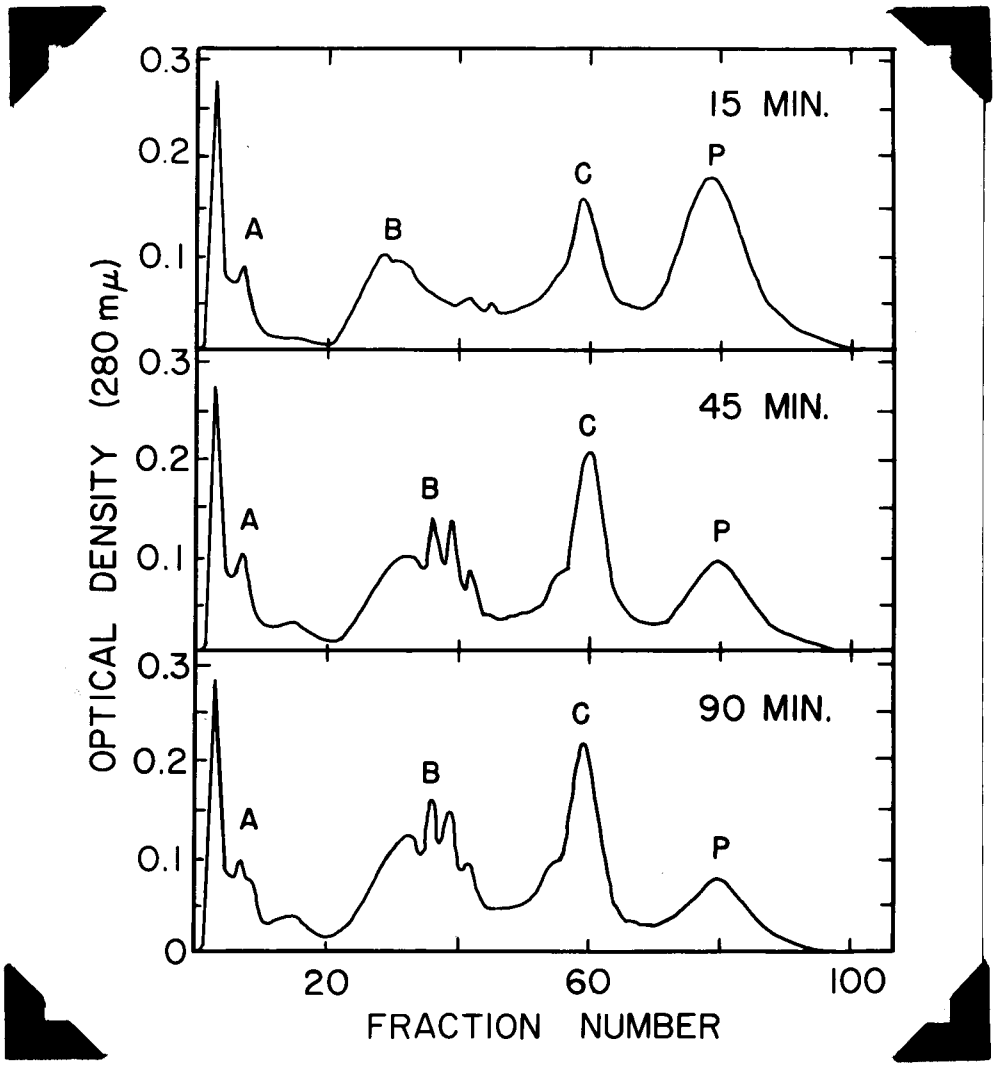


FIG. 7

THE CHROMATOGRAPHY OF ALKALINE INACTIVATED PEPSIN

Experiment #167, #169, #172A: 50 mg. pepsin in 2.0 ml. 0.10 M phosphate buffer at pH 6.7, 23°, for the indicated length of time.

protein in the "P" component is accompanied by the appearance of a component which is eluted maximally at Fraction #59. A similar peak was found to be present when pepsin was incubated at pH 5.8, 4.0 and 2.5 in 8 M urea solution prior to chromatography (Fig. 5). This component has also been labeled "C", and its area has been separated from component "B" by the same procedure used for the chromatograms in Fig. 5. A peak at Fraction #59 can also be detected in the chromatogram of untreated pepsin shown in Fig. 1. After 45 minutes at pH 6.7, component "C" accounts for approximately one-fourth of the total chromatogram absorption (Table XVII). The increased formation of component "A" is not due to an increase in the protein material eluted maximally at Fraction #3, as is the case when pepsin is incubated in 8 M urea at pH 5.5. The optical density of Fraction #3 of the pH 6.7 chromatograms remains constant up to 90 minutes incubation of the pepsin sample. The increase measured in component "A" is apparently due to an increase in the protein material from Fraction #7 to #20.

The data of Table XVII has been plotted in Fig. 8. The major differences between urea inactivation and pH 6.7 inactivation can be seen by comparing Fig. 4 with Fig. 8. The total increase of components "A" and "B+C" are markedly different.

The peaks in the "B" component of the pH 6.7 treated pepsin chromatograms appear to be qualitatively similar to those of the "B" component of the urea treated pepsin chromatograms.

TABLE XVII

THE EFFECT OF INCUBATION ON THE INACTIVATION OF PEPSIN AT pH 6.7*

Expt. No.	Incubation Time	% Total Absorption				Pepsin**
		"A"	"B"	"C"	"P"	
143	None (pH 5.5)	7.0%	23.6%	---	69.4%	100 %
167	15 Min.	14.8	26.0	20.8	38.4	55.4
169	45 Min.	19.5	31.5	27.7	21.3	30.8
172	90 Min.	21.6	35.6	26.7	16.1	23.2

*2.5 gm. % pepsin solution in 0.10 M phosphate buffer, 23°.

**The pepsin ("P") component of Experiment #143 is taken as 100%.

The value for fraction "B" in Experiment #143 includes the area denoted by "C".

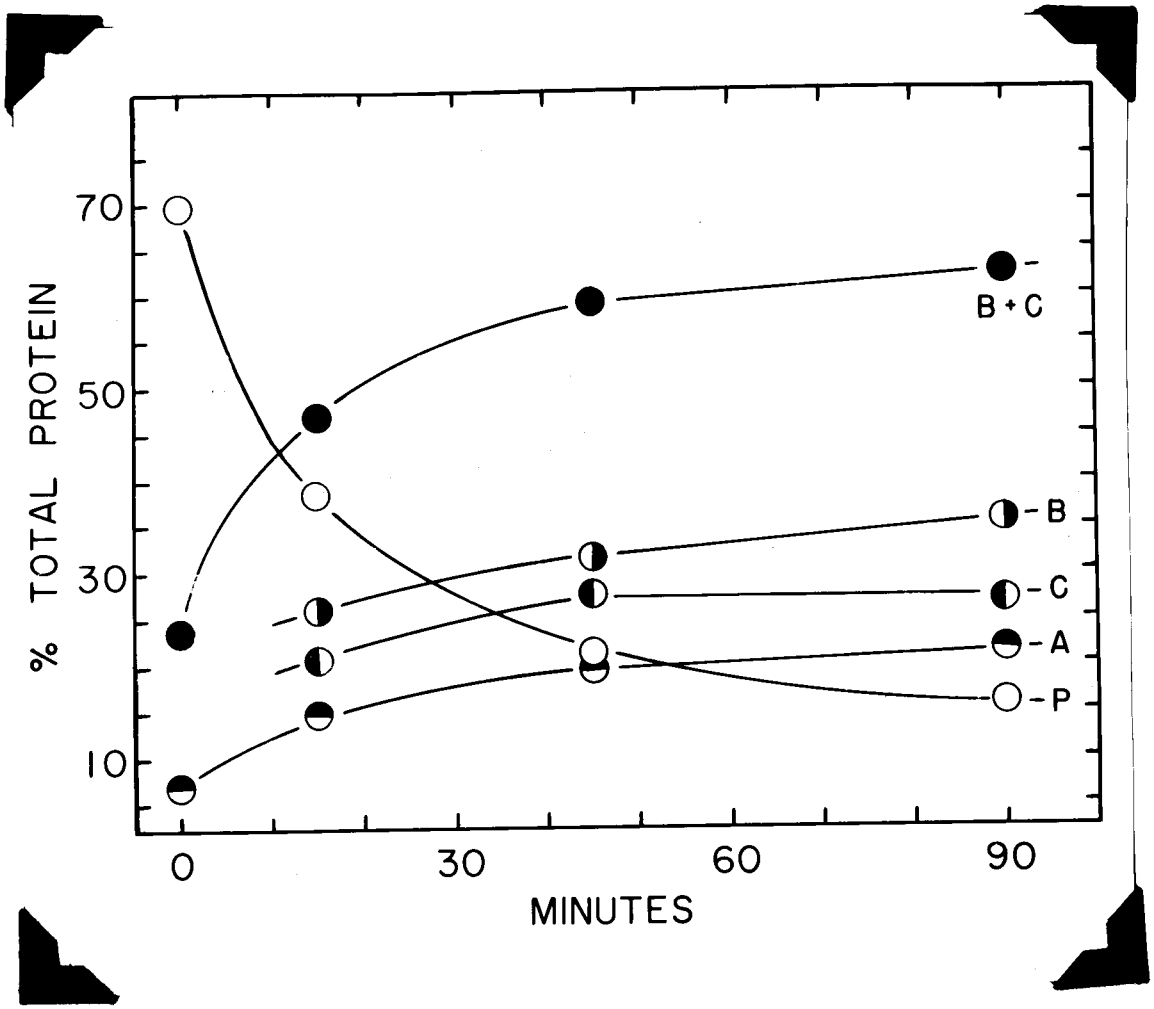


FIG. 8

THE INACTIVATION OF PEPSIN AT pH 6.7 AND THE FORMATION OF NON-PEPSIN PROTEIN MATERIAL

Pepsin was incubated in 0.10 M phosphate buffer at pH 6.7, 23°, prior to chromatography. Components were measured by planimetry of the area under the chromatographic elution patterns. (see Fig. 7 and Table XVII)

A comparison of the two series of chromatograms will be given in the following section.

D. Comparison of the Effects of Urea and Alkaline Inactivation

The difference between the urea and alkaline inactivation of pepsin can be seen by comparing the chromatograms presented in Figs. 3 and 7. Fig. 9 points up these differences more sharply. Fig. 9A (solid line) is the pattern obtained from a sample of pepsin that has been treated in 8 M urea-acetate buffer at pH 5.5, 37°, for 60 minutes. Fig. 9B (solid line) is the chromatogram of a pepsin sample treated in pH 6.7 phosphate buffer at 23° for 45 minutes. The "P" component represents 19 per cent of the total protein in Fig. 9A, and 21 per cent of the total protein in Fig. 9B, so that reasonable comparisons can be made between the two chromatograms. The dotted curves in Fig. 9 are the color values of the fractions obtained with the Folin's phenol reaction for tyrosine and tryptophane. As pointed out previously, the two methods give different results for a protein, depending on the ratio of tyrosine and tryptophane contents.

The presence of the large peak in component "C" of Fig. 9B, as opposed to the larger amount of total material in component "A" of Fig. 9A, is the major difference between the two types of inactivation. The comparison of the chromatograms in Fig. 9 also demonstrates the gross differences in the "B" components.

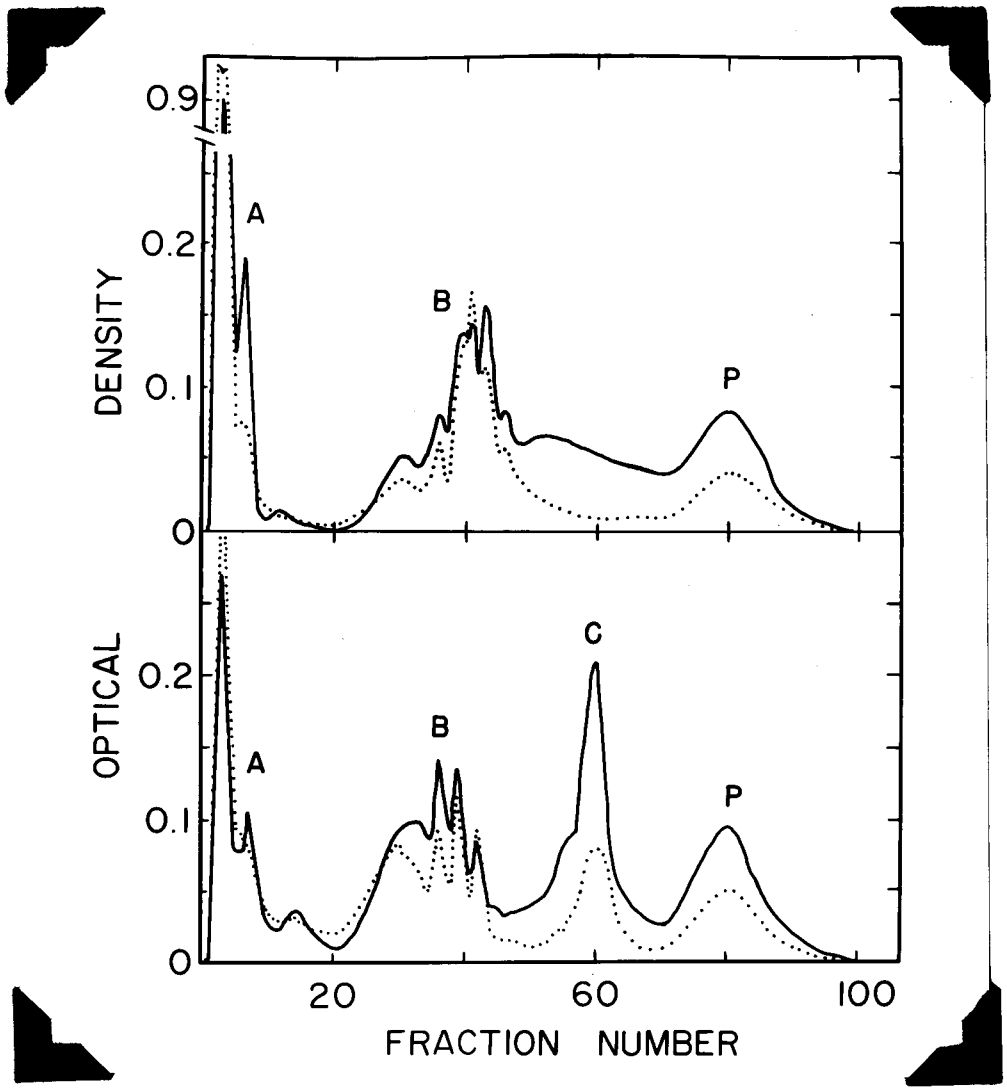


FIG. 9

COMPARISON OF THE EFFECT OF UREA AND ALKALINE INACTIVATION OF PEPSIN

Fig. 9A - Experiment #158: 50 mg. of pepsin in 5.0 ml. of 8 M urea-acetate at pH 5.5, 37°, for 60 minutes.

Fig. 9B - Experiment #169: 50 mg. of pepsin in 2.0 ml. of 0.10 M phosphate buffer at pH 6.7, 23°, for 45 minutes.

The pH, chloride ion concentration, and maximum absorption (280 mu) of the peaks of both chromatograms in Fig. 9 are listed by their fraction number in Table XVIII. The ratio of the optical density of the fraction (280 mu) to the Folin's reaction color value of the fraction is given in the last two columns. These ratios are found to vary considerably throughout the chromatogram, but similar ratios between two peaks (of the same or different chromatogram) should suggest a similarity of tyrosine/tryptophane ratio.

The data of Table XVIII shows that only five peaks, excluding pepsin (#80), coincide exactly by fraction number. The two major peaks of component "A", Fraction #3 and #7, are found to coincide in both chromatograms. However, the optical density of Peaks #3 and #7 in component "A" of the chromatogram in Fig. 9A, is much greater than the same peaks of component "A" in Fig. 9B. The O.D./Folin color value ratio of Peak #3 is identical in both chromatograms, indicating a similarity of tyrosine/tryptophane content. The ratios for Peak #7 are different.

In the "B" component of both chromatograms, Fractions #30, #36 and #39 are found to be peak fractions, but only Fraction #39 of both chromatograms has similar optical density reading. Peak #30, of Experiment #169, has been distorted by the presence of the adjacent peak in Fraction #32, but it

TABLE XVIII

PEAKS FOUND IN CHROMATOGRAMS OF UREA AND ALKALINE INACTIVATED PEPSIN

Fraction Number	Cl^-	pH	Peak Fractions (Optical Density)		O.D./Folin Color	
			#158	#169	#158	#169
3 "A"	0	5.50	0.900	0.270	0.8	0.8
7	0	5.50	0.182	0.105	2.3	1.3
14	0	5.50	-----	0.036	1.1	1.1
30 "B"	0.060	5.00	0.052	0.096*	1.4	1.1
32	0.075	4.91	-----	0.099*	1.4	1.4
36	0.095	4.75	0.080	0.140	1.4	1.5
39	0.109	4.65	0.135	0.135	1.2	1.1
41	0.117	4.59	0.143	-----	0.9	1.4
42	0.122	4.55	-----	0.084	1.0	0.9
43	0.126	4.52	0.156	-----	1.4	1.0
46	0.137	4.44	0.085	-----	1.6	2.1
55 "C"	0.163	4.25	-----	0.089*	4.5	3.4
59	0.173	4.18	-----	0.208	6.0	2.6
80 "P"	0.209	3.91	0.085	0.094	2.1	2.0

Expt. #158 - 51.0 mg. pepsin in 5.0 ml. 8 M urea-0.1 M acetate at pH 5.5, 37°, for 60 minutes. (18.9% component "P" present)

Expt. #169 - 49.7 mg. pepsin in 2.0 ml. 0.10 M phosphate at pH 6.7, 23°, for 45 minutes. (21.3% component "P" present)

*Peak not completely resolved.

is clearly ascertained by the Folin's phenol reaction (Fig. 9B, dotted line), since the tyrosine/tryptophane ratio of the material in Fraction #30 is obviously greater than that of Fraction #32. The O.D./Folin color ratio of Peak #36 is the same in both chromatograms. The same is true for Peak #39. The coincidence of fraction numbers and the identical O.D./Folin color ratios of Peaks #36 and #39 in both chromatograms suggest a partial similarity of the products of urea inactivation with the products of the alkaline inactivation of pepsin.

It should be noted that in all the chromatograms run on pepsin samples treated with 8 M urea at pH 5.5, all the detectable peaks of the chromatograms corresponded to the fraction numbers listed in Table XVIII, with the exception of Peaks #30 and #80 (the pepsin peak). The same is true for the series of chromatograms run on pepsin samples incubated at pH 6.7, with the exception of Peak #59 (which appeared in Fraction #60 in the 45 minute chromatogram), and Peak #80 (the pepsin peak). Because of this excellent reproducibility, emphasis can be placed on the slight differences in fraction number of the peaks found in the two series of chromatograms, as given in Table XVIII. In all probability, Peak #42 of the pepsin chromatogram treated at pH 6.7 (Experiment #169), is truly chromatographically distinct from Peaks #41 and/or #43 of the chromatogram of pepsin treated with 8 M urea at pH 5.5 (Experiment #158).

The position of the pepsin peak varied from Fraction #78 to #81 in both series of chromatograms. This variability of the elution fraction of the pepsin can be explained on the basis of the rate of change of the gradient buffer in this area of the chromatogram. The rate of change of chloride ion concentration and pH in the vicinity of Fraction #80 is extremely small. The change from Fraction #78 to #81 is found to be only about 0.003 M chloride (0.207 M to 0.210 M), and 0.03 pH units (pH 3.93 to 3.90). In the earlier part of the chromatogram, the rate of change of chloride concentration and pH is much greater (Table XVIII).

In order to obtain some indication of the approximate molecular size of the breakdown products formed during the inactivation of pepsin, a sample of pepsin was treated at pH 6.7, 23°, for 90 minutes (Experiment #172A and #172B). One-half of the sample (5.0 ml.) was chromatographed immediately, while the other half was dialysed at 6° against one liter of 0.10 M acetate buffer at pH 5.5, for 26 hours, prior to chromatography. The results of this experiment are shown in Fig. 10. The dialysed preparation is shown by the dotted line. Immediately, it can be seen that all of component "P", and most of component "C" is non-dialysable, whereas most of components "A" and "B" are dialysable. The only distinguishable peak found in component "B"

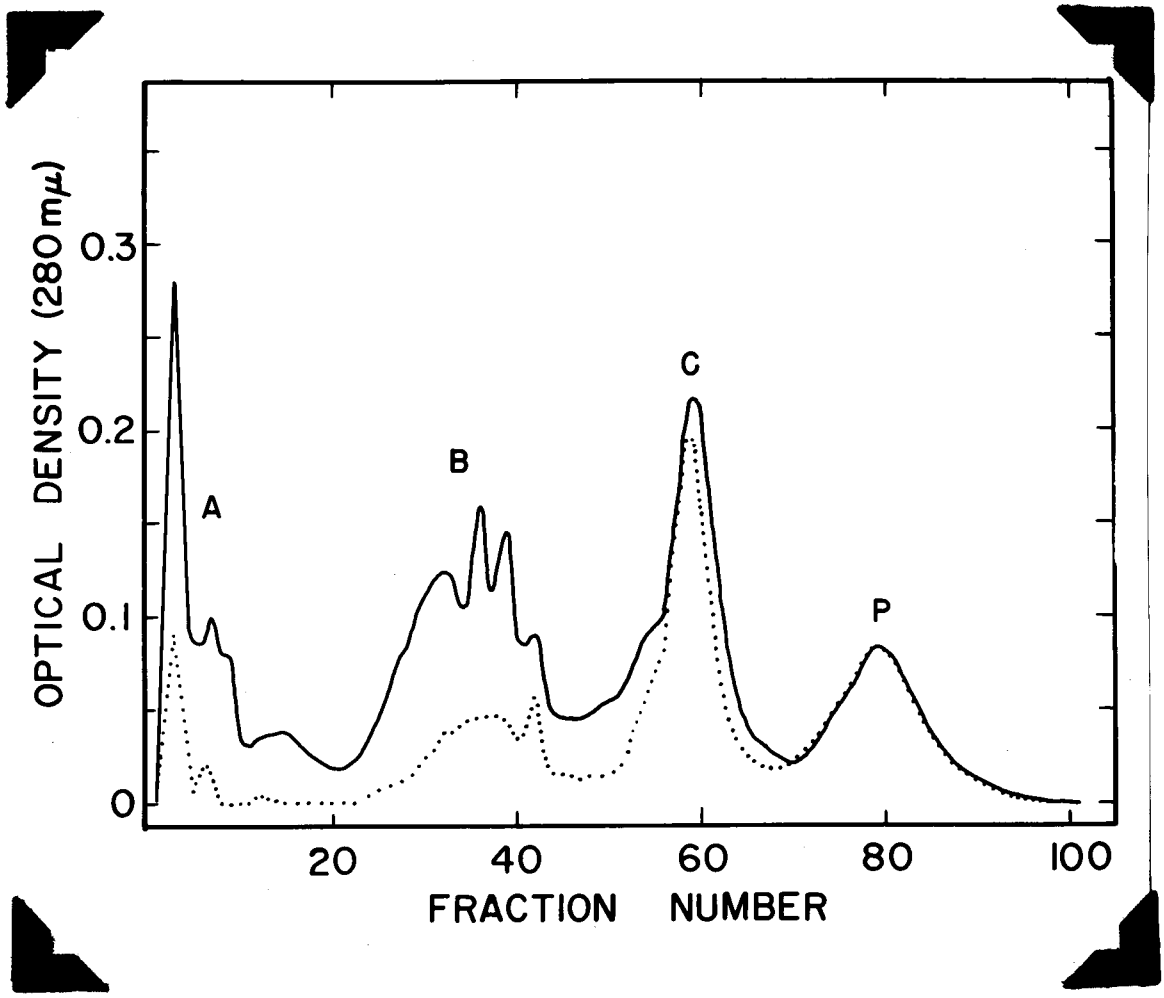


FIG. 10

DIALYSIS OF THE PRODUCTS OF PEPSIN INACTIVATION

Experiment #172A: (solid line) Chromatogram of pepsin incubated for 90 minutes at pH 6.7, 0.10 M phosphate, 23°. Experiment #172B: (dotted line) Same sample as Experiment #172A, dialysed against one liter 0.10 M acetate buffer pH 5.5, (6°) for 26 hours, prior to chromatography. (See Table XIX)

of the dialysed sample is in Fraction #42.

The same dialysis procedure was performed on a sample of pepsin treated in 8 M urea, pH 5.5, 37°, for 155 minutes (Experiments #179A and #179B). The chromatogram of the dialysed preparation (#179B) is qualitatively similar to that of Experiment #179A, shown in Fig. 3. The concentration of all peaks were reduced, except that of Fractions #36 and #80. The absorption of Fraction #36 (and the surrounding fractions) of the dialysed preparation was greater than the absorption of the corresponding fraction in the non-dialysed preparation. This may indicate that urea somehow interferes with the chromatography of some of the breakdown products. The planimetry results of both dialysis experiments are given in Table XIX.

E. Inactivation of Pepsin Above pH 6.7

When pepsin is inactivated in aqueous solutions more alkaline than pH 6.7, peculiar chromatographic results are obtained. Fifty milligrams of pepsin was dissolved in 2.0 ml. of pH 7.5, 0.09 M phosphate buffer. The final pH was 7.0. After 15 minutes at 23°, the solution was acidified to pH 5.5, diluted to 5.0 ml. and chromatographed. The chromatogram obtained is indicated by the solid line in Fig. 11A. Approximately two per cent of the total protein is pepsin. The "A" component comprises about eight per cent of the total, just slightly more

TABLE XIX

DIALYSIS OF THE PRODUCTS OF PEPSIN INACTIVATION

Chromatogram Component	% Total Absorption			
	Experiment #179		Experiment #172	
	Before Dialysis	After Dialysis (24 hrs)	Before Dialysis	After Dialysis (26 hrs)
"A"	46.5 %	10.7 %	21.6 %	3.5 %
"B"	28.3	12.3	35.6	11.5
"C"	17.2	9.2	26.7	19.2
("B + C")	(45.5)	(29.3)	(62.3)	(30.7)
"P"	8.0	8.0	16.1	16.1
Total	100 %	40.2 %	100 %	50.3 %

Experiment #179 - Chromatography of pepsin treated in 8 M urea-0.1 acetate at pH 5.5, 37°, for 155 minutes.

Experiment #172 - Chromatography of pepsin treated at pH 6.7, 0.1 M phosphate, 23° for 90 minutes.

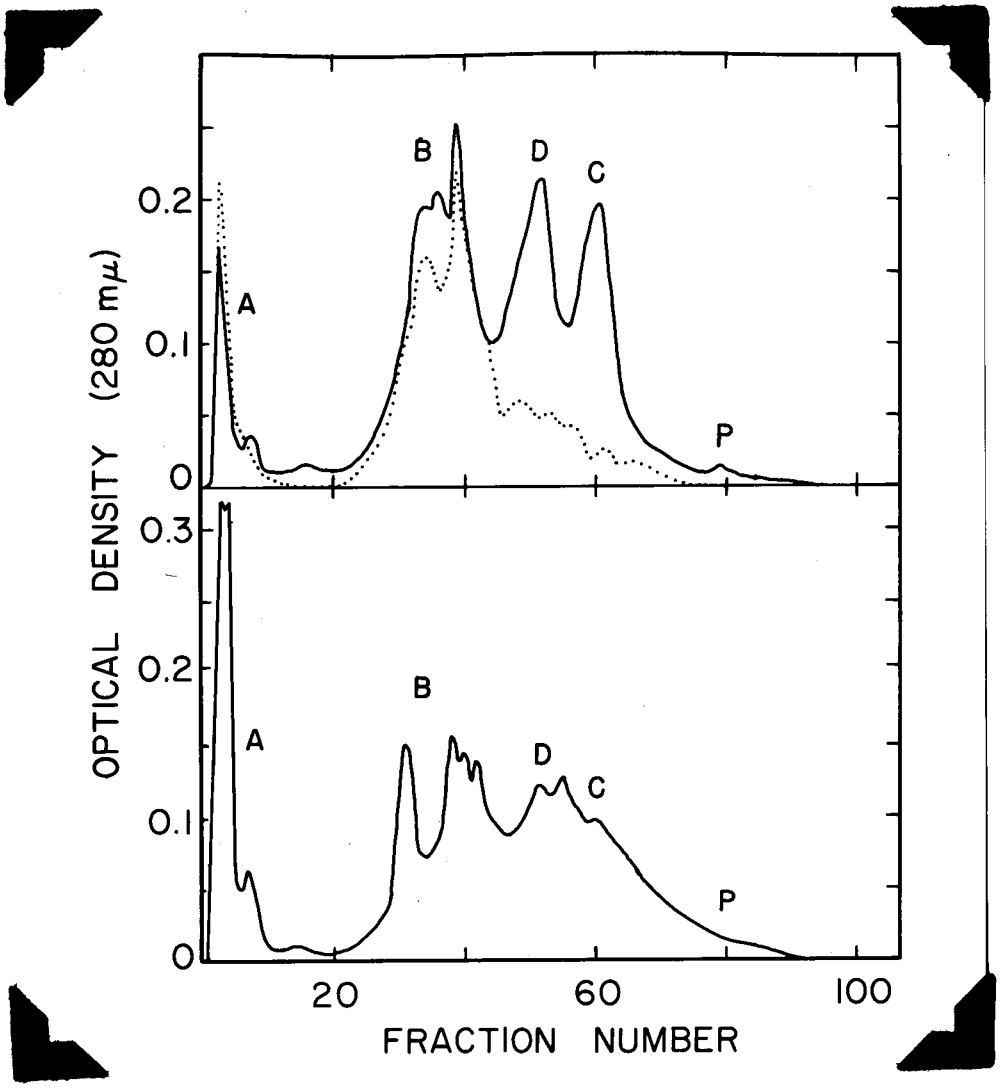


FIG. 11

EFFECT OF UREA ON THE CHROMATOGRAPHY OF THE INACTIVATION PRODUCTS OF PEPSIN

Fig. 11A - Experiment #177: Solid line - chromatogram of pepsin incubated for 15 minutes in 0.09 M phosphate buffer at pH 7.0, 23°. Dotted line - TCA soluble material.

Fig. 11B - Experiment #178: Pepsin was treated identical to Experiment #177, then urea and acid added to give a final solution of 8 M urea, pH 5.5, prior to chromatography.

than is found in this component when pepsin is chromatographed without treatment of any kind. The appearance of another peak between components "B" and "C" is most conspicuous. This peak appears in Fraction #52, and has been labeled "D" in Fig. 11A. Components "C" and "D" have each been estimated to be approximately one-fifth of the total protein.

The fractions of the chromatogram shown in Fig. 11A were tested for TCA solubility. The dotted line indicates the TCA soluble protein material of each fraction. Practically all of component "A" is soluble, and it appears that all but a single protein in component "B" is also soluble in ten per cent TCA. Component "B" has peak absorption in Fractions #34, #36 and #39, prior to treatment with TCA. Peak #36 disappears after the fractions are treated with TCA. The measurement of the per cent absorption for this experiment (#177), is given in Table XX. It can be seen that the disappearance of Peak #36 corresponds to a loss of about ten per cent of the total absorption from component "B". Practically all of components "C + D" are insoluble in TCA, indicating that they probably have molecular weights similar to pepsin.

When pepsin is incubated at a pH above 7.5, it was not found possible to recover all of the protein from the column. This happens whether or not urea is present in the buffer. When pepsin is treated at pH 8.0 prior to chromatography, only 55 to

TABLE XX

TCA SOLUBILITY AND THE EFFECT OF UREA ON THE CHROMATOGRAPHY OF THE INACTIVATION PRODUCTS OF PEPSIN

Expt. No.		% Total Absorption			
		"A"	"B"	"C & D"	"p"
177*	Total Chromatogram	7.9%	45.5%	44.4%	2.2%
177	TCA Soluble Material	6.8	34.0	11.4	0
178**	8 M Urea Added	19.7	40.0	37.0	3.3

*50 mg. pepsin in 2.0 ml. 0.09 M phosphate buffer, pH 7.0. After 15 minutes at 23°, sample was acidified to pH 5.5, diluted to 5 ml. and chromatographed.

**Pepsin was treated in a manner identical to Experiment #177. Urea and acetic acid were added to give a final solution of 8 M urea, pH 5.5 (10 ml. final volume) immediately prior to chromatography.

65 per cent of the protein can be eluted from the column. When the pepsin is first dissolved in 0.3 M NaOH, then acidified to pH 5.5 and applied to the column, only 28 to 30 per cent of the total protein is recovered. The remainder of the protein cannot be eluted with 1 M HCl, nor with 0.10 M acetate buffer, pH 4 - 4.5, containing NaCl up to a concentration of 1.5 M. About five to seven per cent of the total protein applied to the column can be eluted before the gradient eluent is started. This corresponds to component "A". The remainder of the protein is eluted in the area of the chromatogram previously designated as component "B". This protein was not eluted in a definite pattern.

F. Artifacts Caused by Urea

Urea is primarily considered to be a hydrogen bond breaking reagent. However, it is also known to increase the solubility of some proteins in aqueous solutions. The mechanism of the "solubilizing effect" is not known, but it is conceivable that urea molecules can be adsorbed to protein molecules by hydrogen bonding. The adsorption of urea molecules may effect the chromatography of proteins, thereby causing artifacts to appear in the chromatogram. In order to test this possibility, 50 mg. of pepsin was inactivated at pH 7.0 in 2.0 ml. of 0.09 M phosphate buffer as in Experiment #177. After 15 minutes at 23°,

8.0 ml. of 10 M urea-acetate buffer was added to bring the final concentration to 8 M urea, pH 5.5. The chromatogram of the pepsin solution treated in this fashion is shown in Fig. 11B, and may be compared to Fig. 11A. The planimetry measurements of total absorption are included with Experiment #177 in Table XX.

The effect of the urea was greater than had been anticipated. About 12 per cent of the total absorption has shifted from components "B", "D" and "C", over to component "A". All of the urea in the sample is washed through the column with component "A" in the first five to six fractions. The increase in "A" may then be due to the "solubilizing effect" of urea. The increase in the absorption of Fraction #36 noted when pepsin treated in 8 M urea at pH 5.5 is dialysed before chromatography, may also be a reflection of the "solubilizing effect" of urea (Experiment #179B). This experiment is not strictly comparable to the experiments at pH 5.5, since twice the amount of urea has been used in this experiment, as compared to the amount of urea used in the experiments at pH 5.5.

Besides the increase in component "A", the effect on the peaks is evident. Components "D" and "C" have been virtually fused together, and this material has tailed into component "P". Even more drastic, Peak #36, which was shown to be insoluble in ten per cent TCA, has disappeared. The optical density of the

highest peak in Fig. 11A, Peak #39, has been decreased to almost one-half of its original value by the treatment with urea. Furthermore, two new peaks are now to be noted. These peaks are in Fractions #41 and #43; the same peaks that were found exclusively in the chromatograms of the pepsin samples pretreated with urea. It appears probable that these peaks may be artifacts caused by the presence of urea.

G. N-Terminal Amino Acids

The "P" component from chromatograms of pepsin treated in 8 M urea at pH 5.5, and in phosphate buffer at pH 6.7, were analysed for N-terminal amino acids by the DNFB method. The "C" component from a chromatogram of pepsin incubated at pH 6.7 was also analysed. In all cases the results were similar to those found previously for the pepsin sample (Table IX). Isoleucine was present in approximately equimolar amounts. Small amounts of aspartic acid, glutamic acid, serine, alanine, glycine, threonine and valine were also found to be present. The "A" and "B" components, of the chromatograms were not analysed.

There was no increase in any of the N-terminal amino acids when pepsin was incubated at pH 6.7 prior to dinitrophenylation. The analysis could not be performed on a sample of pepsin incubated in 8 M urea at pH 5.5. The presence of urea caused the formation of large amounts of dinitrophenylamine,

which interferes with the chromatography of the DNP-amino acids.

H. Enzymatic Activity

All chromatographic fractions were not tested routinely for enzymatic activity. The activity of the chromatogram fractions were tested only when the treatment of the pepsin sample differed, or when the chromatographic pattern obtained differed qualitatively from previous patterns. In all chromatograms tested, enzymatic activity toward hemoglobin was only found in the "P" component. The relative specific activity of the "P" component was essentially constant in the area of the component that appeared to be homogeneous chromatographically, and it was found to average from 25 to 27 relative specific activity units.

Table XXI lists the data and the calculations of the "P" components from three chromatograms of pepsin samples that were treated in different ways.

TABLE XXI

RELATIVE SPECIFIC ACTIVITY OF PEPSIN CHROMATOGRAPHIC FRACTIONS

Experiment #143				Experiment #144				Experiment #169			
Tube No.	A	B	A/B	Tube No.	A	B	A/B	Tube No.	A	B	A/B
64	0.119	0.039	3.1	69	0.202	0.030	6.7	67	0.111	0.039	2.9
67	0.296	0.053	5.6	72	1.074	0.063	17.1	70	0.133	0.030	4.4
70	1.856	0.112	16.6	75	3.960	0.154	25.7	73	0.696	0.041	17.0
73	6.114	0.237	25.8	77	5.250	0.220	23.9	76	1.329	0.071	18.7
76	9.130	0.337	27.1	79	6.280	0.237	26.5	78	2.193	0.087	25.2
78	10.100	0.355	28.5	80	6.250	0.227	27.6	80	2.478	0.094	26.3
80	8.620	0.310	27.8	81	5.520	0.204	27.1	82	2.226	0.084	26.5
82	6.714	0.226	29.7	84	3.075	0.115	26.7	85	1.314	0.049	26.8
85	3.436	0.134	25.7	93	0.422	0.017	24.8	88	0.836	0.031	27.0
88	1.718	0.069	24.9	96	0.252	0.011	22.9	91	0.388	0.015	25.9
91	0.940	0.037	25.4	99	0.164	0.007	24.1	94	0.202	0.008	25.3
Average (73-91)			26.9	Average (75-99)			25.3	Average (78-94)			26.1

Experiment #143 - pH 5.5 acetate buffer, 1 Min.

Experiment #144 - pH 5.5, 8 M urea, 37°, 5 Min.

Experiment #169 - pH 6.7, 23°, 45 Min.

A - Increase in optical density of hemoglobin TCA filtrate at 280 mu after incubation with 1.0 ml. of fraction.

B - Optical density of fraction at 280 mu.

A/B - Relative specific activity.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The interpretation of the experimental observations reported in this dissertation depends, to a certain extent, on the reports of other investigators. These findings were discussed in Chapter II, and may be summarized as follows:

1) Pepsin is a straight chain protein with a molecular weight of approximately 34,500 (see Table I).

2) When pepsin is dissolved in acid solution, the enzymatic activity disappears slowly. Since the inactivation is paralleled by an increase in trichloroacetic acid soluble peptides, the loss of activity has been attributed to autodigestion. Pepsin digests most proteins optimally at pH 1.5 - 2. The autodigestion of pepsin is also optimal in this pH range.

3) Pepsin is active in 8 M urea solution, but the enzymatic activity is lost rapidly upon standing at temperatures above 20°. In urea solution below pH 4, pepsin inactivation is accompanied by an increase in trichloroacetic acid soluble peptides (see Tables VI and VII).

4) The "alkaline denaturation" of pepsin (i.e., the inactivation of the enzyme occurring above pH 6) is paralleled

by the formation of acid insoluble protein (see Table IV).

5) Pepsin undergoes gross changes in the "shape" properties of its molecular unit during "alkaline denaturation" (see Table V).

The following conclusions may be drawn from the results of the experiments described in Chapters III and IV:

(A) When pepsin is dissolved in acetate buffer at pH 5.5 and chromatographed on DEAE-cellulose, the enzymatically active protein is recovered as a single, homogeneous peak (component "P") amounting to approximately 70 per cent of the total proteinaceous material (Fig. 1). The inactive material is arbitrarily separated into two parts: component "A" is the material which can be eluted with pH 5.5 acetate buffer; component "B" is the material which is eluted with the gradient buffer. Both components are chromatographically heterogeneous.

The relative specific activity of the "P" component is only about five per cent greater than that of the original pepsin sample. Since the active pepsin comprises only 70 per cent of the total material, it seems likely that some of the pepsin has been inactivated by the chromatographic procedure. This interpretation is favored by the fact that the increase observed in the relative specific activity of the pepsin sample

agrees well with the purity analyses performed on the sample (see p. 38). When pepsin is incubated in acetate buffer at pH 5.5, only a slight loss of activity can be detected. When pepsin is chromatographed in the same buffer, it would seem likely that the greater loss of activity is due to surface denaturation and/or autodigestion of the active protein on the column. The "A" component is effeciently eluted from the column in the first five fractions. In all probability it is present in the sample at the time that the sample is applied to the column. The "B" component, however, is devoid of peaks, and it would appear that most of this component is continuously formed and eluted from the strongly adsorbed "P" component during developement of the chromatogram.

(B) If pepsin is incubated in acid solution prior to chromatography, the active pepsin component decreases, while component "A" shows a concomitant increase. Component "B" remains essentially constant (Fig. 2). This result is presumed to be due to pepsin autolysis. The protein of component "P" is apparently degraded to small peptide products which chromatograph in the "A" component.

(C) Pepsin activity is rapidly lost in 8 M urea solutions. When pepsin is incubated in 8 M urea at pH 5.5, 37°,

prior to chromatography, the decrease occurring in the "P" component is accompanied by simultaneous increases in both the "A" and the "B" components (Fig. 3). After 155 minutes of incubation, only about ten per cent of the active pepsin remains, and "A" and "B" are present in approximately equal amounts. Component "B" is resolved into at least six chromatographically distinct peaks. The dialysis experiments indicated that both components are dialysable, but the peptides of component "A" appear to be relatively smaller than the peptides in component "B".

The loss of active pepsin in 8 M urea solution can probably be correlated with autolysis. Urea inactivation of pepsin is more rapid than the inactivation normally found in aqueous solution of the enzyme, so that the effect of urea may be a general enhancement of autolysis. This increased autolysis may be due to either an activation of enzyme action by the urea, or to the formation of urea denatured pepsin. In view of the known denaturing action of urea, the latter idea seems more acceptable. The former concept seems improbable since it has been shown that the initial activity of pepsin on protein substrates in urea solution is the same as in aqueous solution. Furthermore, lower concentrations of urea apparently have no effect on the stability of pepsin.

Since proteolytic enzymes are known to digest the

denatured form of a protein more rapidly than the native form, the rapid inactivation of pepsin by urea could best be explained by assuming that pepsin will hydrolyse urea denatured pepsin molecules at a much faster rate than native pepsin molecules. Inactivation would then be due to denaturation of some of the pepsin molecules by urea, followed by digestion of the denatured species by the residual native molecules.

The formation of the peaks in the "B" component during urea inactivation, appears to indicate that the autolysis of urea denatured pepsin is somewhat different than the autolysis of native pepsin. If the peptides formed in component "B" are larger than the peptides formed in component "A", then this might indicate that the "B" peptides are intermediate hydrolysis products which, if subjected to further pepsin action, would yield smaller peptides which would chromatograph in the "A" component.

The autolysis of urea denatured pepsin would differ from the autolysis of pepsin occurring in aqueous solution if urea could, in some way change, or interfere with the specificity of pepsin action. If pepsin has, for instance, two types of "active centers", each with a specificity toward different peptide bonds, urea may inhibit one of the "active centers", but not the other. On the other hand, urea molecules could be adsorbed onto certain peptide bonds of the denatured pepsin

(substrate) molecules, thereby denying the enzyme access to those bonds. The result in either case would be a less extensive autolysis of pepsin with the formation and accumulation of intermediate hydrolysis products.

The postulation that urea is an inhibitor of pepsin action is not the only explanation that can be offered to explain less extensive digestion of pepsin in the presence of urea. It has been suggested that pepsin is inactivated faster in urea solutions than in aqueous solutions because of the rapid formation of denatured pepsin. This relates to the availability of a "protein species" that is more susceptible to enzymatic hydrolysis. It must be kept in mind, that the denatured or inactive "protein species", is made available at the expense of the native, or active enzyme. In acidic solution, autodigestion probably proceeds through the formation of a "protein species" which is more susceptible to enzymatic hydrolysis. This "protein species" may be intact pepsin molecules which have somehow been denatured by the environment, or by other native pepsin molecules, or it may be a protein and/or peptide that has been formed from the hydrolysis of a single peptide bond in a native pepsin molecule. In either case, the formation of this species is the rate determining step of the reaction, leading to complete autolysis of a protein molecule. That is, as soon as this step is accomplished, the remaining susceptible bonds of the newly formed

molecule are hydrolysed readily, leading to the formation of large amounts of end products, and at the same time preventing a build up of the larger, intermediate hydrolysis products. Thus pepsin autolysis in acid solution would appear to be an "all or none" reaction, where all of the susceptible bonds in a pepsin molecule are attacked and hydrolysed at the same time. The appearance of an "all or none" type of reaction can be considered to be due to the presence of excess enzyme. The formation of intermediate products cannot be detected because the concentration of the enzyme greatly exceeds the concentration required for complete hydrolysis of the substrate.

When a protein denaturant such as urea is present, the rate limiting step of proteolysis is removed. The susceptible "protein species" is formed in greater amounts, and the "all or none" type of reaction does not occur. The concentration of the substrate (denatured pepsin) rapidly approaches, and then exceeds, the concentration of the enzyme (native pepsin). The result is the formation and accumulation of appreciable quantities of intermediate hydrolysis products.

(D) Pepsin activity is rapidly lost in aqueous solution at pH 6.7, 23°. The chromatographic patterns demonstrate that the disappearance of the active pepsin is paralleled by increases in components "A", "B" and "C" (Fig. 7). When pepsin

is inactivated at pH 7.0, 23°, component "A" does not increase at all, and another component ("D") appears in the chromatograms (Fig. 11A). Dialysis and TCA solubility experiments indicate that components "C" and "D" are larger than the fractions of components "A" and "B". Component "C" was found to have the same N-terminal amino acid as component "P", indicating that "C" is probably a degradation product from the N-terminus of pepsin. Since pepsin is considered to be a straight chain protein, it appears that some enzymatic hydrolysis has occurred during the inactivation.

When pepsin is incubated above pH 7.5, the chromatographic recovery of protein is poor. The more alkaline the buffer, the smaller the recovery. This may be due to the formation of acid insoluble pepsin, previously characterized by Northrup (56). The conditions of chromatography were such, as to allow precipitation of the "alkaline denatured" protein to occur on the column. If the denatured form of pepsin cannot be recovered in the chromatographic system, then it must be concluded that all of the chromatographic components obtained from the pepsin samples incubated at pH 6.7 and 7.0 are products of pepsin autolysis. Furthermore, since the chromatographic recovery of protein from a sample incubated at pH 7.0 was approximately 100 per cent, it seems likely that all of the "alkaline denatured" pepsin species has been at least partially degraded

to prevent precipitation from occurring. This implies that pepsin has considerable activity at pH 7.0, even though it has optimal activity near pH 2.

All of the remarks that were applied to the autolysis of pepsin in acidic urea solutions could well be applied to the autolysis of pepsin in aqueous solution at pH 6.7 and 7.0. Large amounts of denatured protein are formed rapidly and then hydrolysed just as rapidly by the residual enzyme. The rate of pepsin inactivation at pH 6.7, 23°, is comparable to the inactivation rate at pH 5.5 in 8 M urea, 37°. The presence of the larger intermediate products in the pH 6.7 solution indicates that less extensive hydrolysis has occurred at pH 6.7, as compared to the hydrolysis occurring at pH 5.5 in urea solution. This difference could be attributed to the difference in pepsin activity in the two solutions. However, the great differences in the experimental conditions of the two experiments prevents a closer scrutiny of the causes involved. It may be that pepsin activity is the same at pH 6.7 as it is at pH 5.5, when both experiments are run at 37°, but the rapid denaturation of pepsin at pH 6.7, 37°, does not permit activity measurements.

Conclusive evidence has not been given to demonstrate that the peptide products formed during urea inactivation at pH 5.5 are also formed during inactivation at pH 6.7 and 7.0. The alteration of the peptide chromatographic properties by urea

precludes any close chromatographic comparison. However, it would seem reasonable to speculate that, assuming urea has no influence on pepsin specificity, the "B" components observed in both chromatograms are hydrolysis products of components "C" and "D", just as the "A" peptides may be hydrolysis products of "B".

The determination of the N-terminal amino acids of a pH 6.7 inactivated pepsin sample is apparently not in accord with a hypothesis of hydrolysis. If hydrolysis does occur at pH 6.7, then a corresponding increase in N-terminal amino acids would be expected. However, the experimental evidence indicates that there is no increase in N-terminal amino acids in a pH 6.7 incubated pepsin sample over that found when pepsin is rapidly inactivated in two per cent solution of sodium bicarbonate. It may be possible that regardless of how fast the pepsin is inactivated, autolysis of some denatured protein occurs.

The present investigation arose originally from attempts to obtain peptides of pepsin that would be amenable to amino acid sequence studies. In an indirect way, it appears that this investigation may have fulfilled the original intent. Whatever the actual cause of the formation of the peptide products of pepsin, we now have peptides which are probably of appropriate dimensions to be utilized for amino acid sequence analysis. In most amino acid studies to date, several

proteolytic enzymes have been required to obtain various size peptides of the protein under investigation. With pepsin, an analysis of the peptides obtained from complete autolysis at pH 3, coupled with analysis of the peptides obtained by autolysis in urea solution and at pH 7 should afford an excellent opportunity to piece together a unique sequence of the amino acid structure of pepsin.

SUMMARY

In a previous investigation, the hydrolysis of pepsin by trypsin was studied in an attempt to obtain peptides of pepsin which might be amenable to amino acid sequence studies. Anomalous results of this investigation led to the study reported in this dissertation. The effect of urea and alkali on the inactivation of pepsin was determined by chromatography on DEAE-cellulose columns.

When pepsin is incubated in aqueous solution below pH 6, the activity is lost slowly with a corresponding formation of small peptides. In urea solutions below pH 6, the autolysis proceeds at a rapid rate with the formation of small and intermediate size peptides. The effect of urea appears to be to enhance autolysis through the formation of denatured pepsin.

Pepsin loses activity spontaneously in aqueous solution above pH 6. This inactivation of the enzyme is known as the "alkaline denaturation" of pepsin. When pepsin is incubated above pH 7.5 at 23°, denaturation of the enzyme occurs rapidly and it is accompanied by the formation of acid insoluble protein. If pepsin is incubated at a lower pH, the denaturation process proceeds at a slow rate and the denatured protein is hydrolyzed

almost as rapidly as it is formed. The results appear to indicate that pepsin has considerable activity at pH 7, even though the optimal range of pepsin activity is from pH 1.5 - 2.

The autolysis of pepsin occurring in aqueous solution at pH 6.7 - 7.0 results in the formation of larger intermediate products than the peptides that are formed during the urea inactivation of pepsin. The results indicate that a spectrum of pepsin peptides of various sizes can be easily obtained for use in a study of the amino acid sequence of pepsin.

BIBLIOGRAPHY

1. Alm, R.S., Williams, R.J.P. and Tiselius, H. (1952) "Gradient Elution Analysis", Acta. Chem. Scand., 6, p. 826.

2. Anfinsen, C.B. and Redfield, R.R. (1956) "The Biological Aspects of Protein Structure", Adv. Protein Chem., 11, p. 49.

3. Anson, M.L. (1939) "The Estimation of Pepsin, Trypsin, Papain and Cathepsin with Hemoglobin", J. Gen. Physiol., 22, p. 79.

4. Anson, M.L. (1953) In Les Proteins, R. Stoops, ed., 9th Solvay Council, Institute International de Chimie Solvay, Brussels, p. 201.

5. Astbury, W.T. (1940) "The Hydrogen Bond in Protein Structures", Trans. Faraday Soc., 36, p. 871.

6. Baker, L.E. (1954) "The Kinetics of the Action of Pepsin on Synthetic Substrates", J. Biol. Chem., 211, p. 701.

7. Beaven, G.H. and Holiday, E.R. (1952) "Ultraviolet Absorption Spectra of Proteins and Amino Acids", Adv. Protein Chem., 7, p.319.

8. Bergman, M., Fruton, J. and Hoffman, F. (1941) "The Specificity of Proteinases", Adv. Enzymol., 1, p. 63.

9. Blumenfeld, O.O. and Perlmann, G.E. (1959) "Ultraviolet Difference Spectra of Pepsin", J. Gen. Physiol., 42, p. 563.

10. Blumenfeld, O.O. and Perlmann, G.E. (1959) "The Amino Acid Composition of Pepsin", J. Gen. Physiol., 42, p. 553.

11. Blumenfeld, O.O., Leonis, J. and Perlmann, G.E. (1960) "The Effect of Guanidine Hydrochloride on Crystalline Pepsin", J. Biol. Chem., 235, p. 379.

12. Brand, E. (1948) In Crystalline Enzymes, 2nd ed., Northrup, J.H., Kunitz, M. and Herriott, R.M., eds., Columbia Univ. Press, p. 26.

13. Buchs, S. (1954) "Fundamental Observations on the Existence, Extraction and Activation of Gastric Cathepsin", Enzymologia, 16, p. 193.

14. Cohn, C. (1955) "Optical Rotation and Helical Polypeptide Chain Configuration in Collogen and Gelatin", J. Biophys. Biochem. Cytol., 1, p. 203.

15. Crammer, J.L. and Neuberger, A. (1943) "The State of Tyrosine in Egg Albumin and in Insulin as Determined by Spectrophotometric Titration", Biochem. J., 37, p. 302.

16. Dieu, H.A. and Bull, H.B. (1949) "Monolayers of Pepsin and Insulin", J. Am. Chem. Soc., 71, p. 450.

17. Edelhoch, H. (1956) "The Effect of Urea Analogues and Metals on the Rate of Pepsin Denaturation", Biochem. et Biophys. Acta, 22, p. 401.

18. Edelhoch, H. (1956) "The Mechanism of Pepsin Denaturation", J. Am. Chem. Soc., 78, p. 2644.

19. Edelhoch, H. (1957) "The Denaturation of Pepsin. I. Macromolecular Changes", J. Am. Chem. Soc., 79, p. 6100.

20. Edelhoch, H. (1958) "The Denaturation of Pepsin. II. Hydrogen Ion Equilibria of Native and Denatured Pepsin", J. Am. Chem. Soc., 80, p. 6640.

21. Edelhoch, H. (1958) "The Denaturation of Pepsin. III. The Effects of Various Protein Denaturants on the Kinetics of Pepsin Denaturation", J. Am. Chem. Soc., 80, p. 6648.

22. Edman, P. (1950) "The Reaction of Proteins with Phenylisothiocyanate", Acta Chem. Scand., 4, p. 283.

23. Edman, P. (1953) "Notes on the Stepwise Degradation of Peptides via Phenylthiohydantoins", Acta Chem. Scand., 7, p. 700.

24. Flavin, M. (1954) "Threonylphosposerylglutamic Acid Isolated from Pepsin", Federation Proc., 13, p. 208.

25. Flavin, M. (1954) "The Linkage of Phosphate to Protein in Pepsin and Ovalbumin", J. Biol. Chem., 210, p. 771.

26. Frank, H.S. and Evans, M.W. (1945) J. Phys. Chem., 13, p. 507.

27. Freudenberg, E. (1940) "Uber das Kathepsin des Mogensaftes", Enzymologia, 8, p. 385.

28. Heirwegh, K. and Edman, P. (1957) "Purification and N-terminal Determination of Crystalline Pepsin", Biochim. et Biophys. Acta, 24, p. 219.

29. Herriott, R.M. (1935) "Acetylation of Tyrosine in Pepsin", J. Gen. Physiol., 19, p. 283.

30. Herriott, R.M. (1937) "Inactivation of Pepsin by Iodine and the Isolation of Diiodotyrosine from Iodinated Pepsin", J. Gen. Physiol., 20, p. 335.

31. Herriott, R.M. (1938) "Kinetics of the Formation of Pepsin from Swine Pepsinogen and Identification of an Intermediate Compound", J. Gen. Physiol., 22, p. 65.

32. Herriott, R.M. (1941) "Isolation, Crystallization and Properties of Pepsin Inhibitor", J. Gem. Physiol., 24, p. 325.

33. Herriott, R.M. (1952) "Mechanism Of Enzyme Action", Columbia Univ. Press, New York.

34. Herriott, R.M. (1956) "The Active Groups of Pepsin", J. Cell. Comp. Physiol., 47, Suppl. 1, p. 239

35. Hewitt, L. (1927) "Optical Rotatory Power and Dispersion of Proteins", Biochem. J., 21, p. 216.

36. Hoch, H. (1950) "Electrophoretic Heterogeneity of Crystalline Pepsin", Nature, 165, p. 278.

37. Huggins, M.L. (1943) " The Structure of Fibrous Proteins", Chem. Revs., 32, p. 195.

38. Jacobsen, C.F. and Linderstom-Lang, K. (1949) "Salt Linkages in Proteins", Nature, 164, p. 411.

39. Jirgensons, B. (1958) "Optical Rotation and Viscosity of Native and Denatured Proteins. XII Differences between Human and Bovine Albumins", Arch. Biochem. Biophys., 78, p. 227.

40. Kauzmann, W. and Simpson, R.B. (1953) "The Kinetics of Protein Denaturation. I Behavior of the Optical Rotation of Ovalbumin in Urea Solutions", J. Am. Chem. Soc., 75, p. 5139.

41. Kauzmann, W. (1954) In Denaturation of Proteins and Enzymes, Johns Hopkins Press, Balt., Md.

42. Kauzmann, W. (1959) "Some Factors in the Interpretation of Protein Denaturation", Adv. Prot. Chem., 14, p. 1.

43. Kern, H.L. (1953) Doctorate Dissertation, Johns Hopkins Univ., cited in Ref. 33.

44. Laskowski, M. and Scheraga, H.A. (1954) "Thermodynamic Considerations of Protein Reactions. I Modified Reactivity of Polar Groups", J. Am. Chem. Soc., 76, p. 6305.

45. Laskowski, M., Widom, J.M., Mc Fadden, M.L. and Scheraga, H.A. (1956) "Differential Ultraviolet Spectra of Insulin", Biochim. et Biphys. Acta, 19, p. 581.

46. Levy, A.L. (1954) "A Paper Chromatographic Method for the Quantitative Estimation of Amino Acids", Nature, 174, p. 126.

47. Linderstrom-Lang, K. (1952) Lane Medical Lectures: Proteins and Enzymes, p. 58, Stanford Univ. Press, Stanford, Cal.

48. Linderstrom-Lang, K. and Schellman, J.A. (1955) "The Structure of Insulin as Compared to that of Sanger's A-Chain", Biochim. et Biophys. Acta, 15, p. 156.

49. Merten, R., Schramm, G., Grassmann, W. and Hannig, K. (1952) "Untersuchungen zur Reindarstellung des Mogenkathepsins", Hoppe-Seyl. Z 289, 173.

50. Michaelis, L. and Davidsohn, H. (1910) "Die Isoelectrische Konstante des Pepsins", Biochem. Z. 28, p. 1.

51. Moore, S. and Stein, W.H. (1954) "A Modified Ninhydrin Reagent for the Photometric Determination of Amino Acids and Related Compounds", J. Biol. Chem., 211, p. 907.

52. Neurath, H., Cooper, G.R. and Erikson, J.O. (1941) "The Shape of Protein Molecules", J. Biol. Chem., 138, p. 411.

53. Neurath, H., (1944) "The Chemistry of Protein Denaturation", Chem. Revs., 34, p. 157.

54. Northrup, J.H. (1930) "Crystalline Pepsin. I & II", J. Gen. Physiol., 13, p. 739 - 780.

55. Northrup, J.H. (1946) "Crystallization of Pepsin from Alcohol", J. Gen. Physiol., 30, p. 177.

56. Northrup, J.H. and Kunitz, M. (1948) Crystalline Enzymes, 2nd ed., Columbia Univ. Press, New York.

57. Orekovich, V.N. and Shpikiter, V.O. (1956) Doklady Akad. Nauk SSR, 111, p. 401.

58. Pairent, F.W. (1959) "Tryptic Hydrolysis of Pepsin", Master's Thesis, Loyola Univ., Chicago.

58A. Pairent, F.W. and Williamson, M.B. (1960) Unpublished data.

59. Pairent, F.W. and Williamson, M.B. (1960) "A Modified Two-Dimensional System for the Paper Chromatography of DNP-Amino Acids", J. Chrom., 4, p. 80.

60. Passman, J.M. (1952) "Studies on the Structure of Pepsin", Master's Thesis, Loyola Univ., Chicago.

61. Passman, J.M. (1954) "The Structure of Pepsin", Doctorate Dissertation, Loyola Univ., Chicago.

62. Pauling, L., Corey, R.B. and Branson, H.R. (1951) "The Structure of Proteins: Two Hydrogen Bonded Helical Configurations of the Polypeptide Chain", Proc. Natl. Acad. Sci. U.S., 37, p. 205.

63. Perlmann, G.E. (1952) "The Phosphorus in Pepsin and Pepsinogen", J. Am. Chem. Soc., 74, p. 6308.

64. Perlmann, G.E. (1954) "Formation of Enzymatically Active, Dialyzable Fragments During Autodigestion of Pepsin", Nature, 173, p. 406.

65. Perlmann, G.E. (1955) "The Nature of the Phosphorus Linkages in Phosphoproteins", Adv. Protein Chem., 10, p. 1.

66. Perlmann, G.E. (1956) "Effect of Urea on Pepsin", Arch. Biochem. Biophys., 65, p. 210.

67. Perlmann, G.E. and Mycek, M.J. (1958) "Modification of Pepsin by Autodigestion", In Symposium on Protein Structure, p. 179, A. Neuberger, ed., John Wiley & Sons, New York.

68. Perlmann, G.E. (1959) "Effect of Solvents and Temperature on the Optical Rotatory Properties of Pepsin", Proc. Natl. Acad. Sci., 45, p. 915.

69. Peterson, E.A. and Sober, H.A. (1956) "Chromatography of Proteins. I Cellulose Ion Exchange Adsorbants", J. Am. Chem. Soc., 78, p. 751.

70. Philpot, J.St.L. and Eriksson-Quensel, I.B. (1933) "An Ultracentrifugal Study of Crystalline Pepsin", Nature, 132, p. 932.

71. Philpot, J.St.L. (1935) "CCXII. The Behavior of Pepsin in the Ultracentrifuge After Alkaline Inactivation", Biochem. J., 29, p. 2458.

72. Pimentel, G.C. and McClellan, A.L. (1960) In The Hydrogen Bond, Chapter 10, p. 298, W.H. Freeman and Co., San Francisco, Cal.

73. Pope, C.G. and Stevens, M. (1951) "The Action of Proteolytic Enzymes on the Antitoxins and Proteins in Immune Sera", Brit. J. Exptl. Path., 32, p. 314.

74. Porter, H. and Sanger, F. (1948) "The Free Amino Group of Hemoglobins", Biochem. J., 42, p. 287.

75. Putnam, F. (1953) In The Proteins, Vol. I., Part B, Chapter 9, H. Neurath and K. Bailey, eds., Academic Press Inc., New York.

76. Rich, A. and Crick, F.H.C. (1955) "The Structure of Collogen" Nature, 176, p. 915.

77. Richmond, V., Tang, J., Wolf, S., Trucco, R.E. and Caputta, R. (1959) "Chromatographic Isolation of Gastricin, the Proteolytic Enzyme from Gastric Juice with pH Optimum 3.2", Biochim. et Biophys. Acta, 29, p. 453.

78. Ryle, A.P. and Porter, R.R. (1959) "Parapepsins: Two Proteolytic Enzymes Associated with Porcine Pepsin", Biochem. J., 73, p. 75.

79. Ryle, A.P. (1960) "Parapepsinogen II: The Zymogen of Parapepsin II", Biochem. J., 75, p. 145.

80. Sanger, F. (1945) "The Free Amino Groups of Insulin", Biochem. J., 39, p. 507.

81. Schellman, J.A. (1953) "The Application of the Bjerrum Ion Association Theory to the Binding of Anions", J. Phys. Chem., 57, 472.

81a. Stark, G.R., Stein, W.H.aand Moore, S. (1960) "Reactions of the Cyanate Present in Aqueous Urea with Amino Acids and Proteins", J. Biol. Chem., 235, p. 3177.

82. Scheraga, H.A. (1957) "Tyrosyl-carboxylate Ion Hydrogen Bonding in Ribonuclease", Biochim. et Biophys. Acta, 23, p. 196.

83. Steinhardt, J. (1937) "The Stability of Crystalline Pepsin", K. Danske Vidensk. Selsk. Mat-fys. Medd., 14, No. 11.

84. Steinhardt, J. (1938) "Properties of Hemoglobin and Pepsin in Solutions of Urea and other Amides", J. Biol. Chem., 123, p. 543.

85. Steinhardt, J. (1939) "Solubility of Crystalline Pepsin. I Anomalous Dependence of Solubility on the Acid to Solvent Ratio", J. Biol. Chem., 129, p. 135.

86. Taborsky, G. (1959) "Chromatography of Ribonuclease on Carboxylmethyl Cellulose Columns," J. Biol. Chem., 234, p. 2652.

87. Tang. J., Wolf, S., Caputto, R. and Trucco, R.E. (1959) "Isolation and Crystallization of Gastricin from Human Gastric Juice", J. Biol. Chem., 234, p. 1174.

88. Taylor, W.H. (1959) "Studies on Gastric Proteolysis. I The Proteolytic Activity of Human Gastric Juice and Pig and Calf Gastric Mucosal Extracts Below pH 5." Biochem. J., 71, p. 73.

89. Tiselius, H., Henshen, G. and Svenson, H. (1938) "Electrophoresis of Pepsin", Biochem. J., 32, p. 1814.

90. VanVunakis, H. and Herriott, R.M. (1957) "The N-terminal of Pepsin and Pepsinogen. The Amino Acid Composition of Pepsinogen", Biochim. et Biophys. Acta., 22, p. 537.

91. VanVunakis, H. and Herriott, R.M. (1957) "The Amino Acid Composition of Pepsin Inhibitor", Biochim. et Biophys. Acta, 23, p. 600.

92. Wetlaufer, D.B. (1956) Compt. rend. trav. lab. Carlsberg, Ser. chim., 30, p. 135.

93. Williamson, M.B. and Passman, J.M. (1952) "The Terminal Amino Group of Pepsin", J. Biol. Chem., 199, 121.

94. Williamson, M.B. and Passman, J.M. (1954) "The Terminal Free Carboxyl Groups of Pepsin", Biochim. et Biophys. Acta, 15, p. 246.

95. Yanari, S. and Bovey, F.A. (1960) "Interpretation of the Ultraviolet Spectral Changes of Proteins", J. Biol. Chem., 325, p. 2818.

96. Yang, J.T. and Doty, P. (1957) "The Optical Rotatory Dispersion of Polypeptides and Proteins", J. Am. Chem. Soc., 79, p. 761.

APPROVAL

The dissertation submitted by Frederick W. Pairent has been read and approved by five members of the faculty of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 22, 1961
Date

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Signature of Adviser